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GAS–LIQUID CHROMATOGRAPHY OF PROTEIN AMINO ACID TRIMETHYLSILYL DERIVATIVES*

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

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SUMMARY

The purpose of the investigation was to make a thorough study of the chemistry of derivatization of the twenty protein amino acids as their N-trimethylsilyl trimethylsilyl (TMS) esters. Major emphasis was directed toward chromatographic separation of the derivatives, precision and accuracy of the method, silylation as a function of reaction temperature and time, molar excess of reactants, stability of the TMS derivatives, quantitative analysis of a synthetic amino acid mixture, and application to biological samples.

The gas–liquid chromatographic separation of the N-trimethylsilyl TMS esters of the twenty protein amino acids was achieved after evaluation of a number of combinations of siloxane liquid phases. The final chromatographic conditions used for the total separation on a single column for all twenty of the amino acids consisted of a mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 coated on high-performance 100/120 mesh Chromosorb G in a 1.75 m × 4 mm I.D. U-shaped borosilicate glass column. Phenanthrene was a suitable internal standard as it was completely resolved from the TMS amino acids. The instrumental settings were 75°, initial hold 7 min, program rate 2°/min, and carrier flow (N_2) of 42 ml/min for fourteen of the amino acids, and 100° initial column temperature for the other six. Prior to chromatography, it is essential to analyze performance blanks under the same chromatographic and instrumental conditions to establish the purity of all chemical reagents.

The reaction conditions were investigated for the quantitative silylation of the twenty amino acids. Fourteen of the amino acids were reproducibly converted to the respective TMS derivatives in 15 min at 135° in a closed vial using a 30 molar excess of bis(trimethylsilyl)trifluoroacetamide (BSTFA)/total amino acids. A comparison of various silylation temperatures showed that silylation at 135° produced the most

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** Professor, Experiment Station Chemical Laboratories.

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§ National Science Foundation Fellow.

reproducible relative molar response values. Also, molar excess studies showed that a 30 molar excess of BSTFA was adequate. This molar excess corresponds to 0.24 ml of BSTFA/mg of amino acid. Acetonitrile was used as a solvent in a 1:1 ratio with BSTFA.

For six of the amino acids (glutamic acid, arginine, lysine, histidine, tryptophan, and cystine) the reaction conditions required were 4 h at 135°. For a complete analysis of all twenty of the protein amino acids, the sample is heated for 15 min at 135° in a closed tube with a teflon-lined screw cap, *cooled within* 3 min, then an aliquot is injected into the gas chromatograph. This analysis provides data for the quantitative determination of alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, hydroxyproline, aspartic acid, methionine, cysteine, phenylalanine, and tyrosine. After injection of the sample, the reaction vial is again tightly recapped, placed in a 135° bath for 4 h, again cooled, and an aliquot injected. After the first injection the column was programmed to about 225° and then cooled to the initial holding temperature of 100° prior to the second injection. This gives the analysis for GLU_2, ARG_4, LYS_4, HIS_3, TRY_3, and CYS_4. The subscripts denote the number of TMS groups attached. The chromatographic time is about 50 min for the six amino acids.

A synthetic known mixture at a 1.0 mg level each of selected amino acids was analyzed to determine the quantitation of the total derivatization and chromatographic method. The accuracy was found to be very good, with recoveries ranging from 93.6 to 105.9%, with an average recovery of 100.8%. The average relative standard deviation (RSD) for the relative molar response values of fourteen of the amino acids was 0.80% and 1.25% for the remaining six amino acids.

The N-trimethylsilyl TMS amino acid esters were found to be completely stable for a period of five to seven days when stored at room temperature in a tightly capped vial. Glycine was the only exception, and deteriorated within a period of 3 h. Glycine changed from GLY_2 to GLY_3. In preliminary experiments, the minimal detectable amount of the TMS derivatives that can be detected in a flame ionization detector at a signal/noise level of 3:1 was found to be 0.3 to 0.5 ng, or 3×10^{-12} moles of each amino acid injected. It is most important that the experimental and instrumental conditions as outlined in this manuscript be followed precisely.

The analysis of ribonuclease as the TMS amino acid derivatives established the applicability of this method for the quantitative analysis of biological materials. The data obtained from the GLC analysis of ribonuclease were found to be in agreement with the values obtained by classical ion-exchange chromatography.

The TMS derivatives of urea, ornithine, citrulline, and ammonium chloride were synthesized and silylated at 135° for both 15 min and 4 h. The second peak for urea increased relative to the first with an increasing reaction time; however, an overall decrease in peak size was noted when silylated for 4 h. On silylation at 150° for 4 h, a further decrease in peak size was noted. Citrulline was found to yield three chromatographic peaks when heated at 135° for 15 min. On heating for 4 h at this temperature, only two peaks were observed, with the higher retention temperature peak being the largest. No chromatographic peaks were observed for ammonium chloride when silylated under any of the above experimental conditions. A complete silylation of ornithine required reacting at 135° for 4 h.

INTRODUCTION

The amino acids have been the subject of intense study by biochemists, nutritionists, medical scientists, biologists, bacteriologists, and investigators in many other areas of science during the past ten years. The literature in this field is immense and provides broad evidence of the striking advances which have been made since the isolation of the first amino acids, asparagine and cystine, about 160 years ago. Since this time, the existence of more than 170 amino acids has been reported, most of them in the last decade. The research on amino acids has been mainly directed toward: (a) a consideration of the amino acids which occur in nature and the forms in which they have been found; (b) observations resulting from nutritional studies; (c) the metabolism of the amino acids, their synthesis, degradation, and the relationships to other metabolites; (d) studies on abnormalities of amino acid metabolism associated with disease states; and (e) protein biosynthesis. In this latter area the scientific advances have been rapid and dramatic during recent years in studies on protein structure. Only some fourteen years separate the reporting by SANGER, Nobel Laureate, of the primary structure of insulin from its complete synthesis almost simultaneously in three different countries.

The increasing activity in this field means that many scientists are faced with the problem of accurate, sensitive, precise, and rapid amino acid analyses.

In the period of 1950 to 1969 the elegant investigations of MOORE, SPACKMAN, STEIN, HAMILTON, PIEZ, and others have developed classical ion-exchange chromatography into a refined method for amino acid analysis. The methods now range from the relatively simple and inexpensive, to the sophisticated, completely automated, and costly.

Also, during the past ten years GLC techniques have reached great sophistication, instruments have become more sensitive and dependable, and there has been a steady flow of reports on the application of GLC to biomedical problems. With such extensive use of GLC for carbohydrates, steroids, lipids, metabolites, and drugs of all kinds, it is at first sight strange that similar methods for the routine analysis of amino acids, compounds of simple structure but of great biochemical interest, have only recently begun to appear. During this same period, research into the functions and behavior of amino acids has formed a fair proportion of the phenomenal research effort that has yielded our present knowledge of the structure of peptides and proteins and of their biosynthesis.

Further, the great upsurge of interest in amino acids and proteins in the last twenty years has led to the development of automated analytical techniques and instrumentation as stated earlier, and their availability has not only taken some of the urgency out of the development of gas chromatographic methods but has also tended to set up resistance to an approach that threatens obsolescence to sophisticated and expensive classical ion-exchange equipment. However, anyone who has used GLC does not need to be convinced of the speed and sensitivity of the method, and any operator of the amino acid analyzer equipment should be further convinced on the grounds of convenience.

The origin of the protein amino acids lies far back in the evolution of life, and chemically they have little in common but their α-carbon atom, with its steric L-configuration depending on the attachment of four different groups. Including these,

there are a dozen different organic chemical functional groups, and it is difficult to devise reaction schemes that will deal successfully with all of these groups with their chemical differences and varying reactivities. However, for a satisfactory analysis by GLC a substantially complete derivatization is necessary. GLC of amino acids has been held back by the lack of agreement on the volatile derivative that should be used. Although there are only about twenty amino acids commonly found in proteins, they have varied chemical structures and it would appear that this would permit an easy separation. This has not necessarily been found to be true, and quantitative derivatization of all the functional groups under a single set of experimental conditions has been most difficult.

The research efforts of GEHRKE, co-workers, and graduate students during the period of 1964–1969 have led to the development of a general quantitative GLC method for the twenty natural protein amino acids and of their complete separation. The derivative of final choice was the N-trifluoroacetyl (N-TFA) *n*-butyl ester. Studies were made on yield, volatility, stability, limit of detection, and general applicability. In our research the following criteria were considered most important for a suitable volatile derivative: (a) no rearrangements or structural alteration should occur during formation; (b) derivatization reaction should be 95 to 100% complete; (c) no sample loss on concentration; (d) stability with respect to time; (e) derivative must have increased volatility; and (f) there must be little or no reactivity of the derivative with the substrate and/or support phase. From this research a good quantitative method was developed, and one which met most of the criteria outlined above. However, there is still need of further simplification of the N-TFA *n*-butyl ester derivatization reaction, for a method which will lend itself to automation, and for a derivative that will be simpler in certain of its chromatographic aspects; as well as for entirely new derivatization and chromatographic approaches.

It was the purpose of this investigation to make a thorough study of the chemistry of derivatization of the twenty protein amino acids as their N-trimethylsilyl TMS esters; emphasis was directed toward precision and accuracy of the method, single column separation, substrate–derivative interaction, recovery, stability of derivative, and application to analysis of amino acids in biological samples.

LITERATURE REVIEW

The trimethylsilyl (TMS) group was introduced by RÜHLMANN AND GIESECKE[1] in 1961 for the GLC analysis of amino acids before BENTLEY *et al.*[2] adopted the TMS group in the carbohydrate field in 1963.

RÜHLMANN AND MICHAEL[3,4] introduced the TMS derivatives into the field of GLC, and reported that after silylation with trimethylsilyldiethylamine (TMSDA) several amino acids could be chromatographed on a column containing 19% silicone oil on Sterchamol. They chromatographed fourteen TMS amino acids, but only valine and phenylalanine were completely resolved, and decomposition was noted for cysteine, lysine, and histidine. Also, RÜHLMANN AND MICHAEL[4] discovered amino acid TMS esters as artifacts in the gas chromatographic analysis of N-TMS amino acid TMS esters, from which they were produced by ammonolysis. The TMS esters (free amino groups) were more volatile than the N-TMS amino acid TMS esters, and a quantitative single-step reaction was devised for their preparation. These authors report quanti-

tative results, but no peaks were obtained for the basic amino acids, presumably because being diamines they would not be sufficiently volatile.

It is well known that gas chromatography of the free bases of amino acid esters is attended by problems associated with the free amino groups. Free amino groups do not lend themselves to GLC and therefore are much better acylated, silylated, or derivatized in some way.

BENTLEY *et al.*[2] dissolved or suspended carbohydrates in pyridine and reacted the mixture with hexamethyldisilazane (HMDS) at room temperature using trimethylchlorosilane (TMCS) as a catalyst, and investigated the analysis of the TMS derivatives of the sugars by GLC.

Until 1965, only a few researchers experimented with the TMS derivatives of the amino acids and with limited success due to the instability of the N-TMS group and the non-availability of an effective silylating reagent. Following the development of some new silylating reagents, studies were renewed on the preparation of the TMS derivatives of the amino acids and it was then considered that the TMS derivatives hold considerable promise as the reaction is complete in one step.

SMITH and coworkers[5,6], in 1965 and 1966, investigated the reaction conditions for the formation of TMS derivatives of amino acids using HMDS and TMCS with different catalysts. They also studied TMSDA, which gave the highest yields (from 89% to 99%, for leucine, serine, and aspartic acid). The boiling points ranged from 76° at 15 mm Hg for alanine, to 147° at 4 mm Hg for lysine. They concluded that TMS diethylamine with some kind of catalyst gave the highest yields of the derivatives of leucine, serine, and aspartic acid. TMS dimethylamine recently has been claimed to react more rapidly and to be more volatile and is recommended for these reasons[7]. BIRKOFER AND DONIKE have suggested the use of N-TMS-N-methyl-acetamide or N-TMS-N-methyl-formamide[8], and KLEBE *et al.* in 1966 reported studies on the synthesis and application of the now widely used N,O-bis(trimethylsilyl)acetamide (BSA)[9] and were able to obtain "sharp single peaks for all the amino acids except arginine, which showed indications of decomposition on the column". Also, the derivatives of glycine and alanine could not be separated from one of the reaction products of BSA, mono(trimethylsilyl)acetamide (MSA), due to its similar chromatographic retention on the SE-30 column they used.

BLAU[10], in a general review of the analysis of amino acids by GLC, cites some further reports on silylation methods by RÜHLMANN AND MICHAEL. They studied the properties of a number of other silylated derivatives[4], *i.e.*, the N-TMS amino acid ethyl esters were not found to be any better for gas chromatography then the N-TMS amino acid TMS esters. Further, an extra step, which was not quantitative, was needed to make them, and this derivative appeared to be less stable and thus was not pursued further. RÜHLMANN AND MICHAEL also tried other trialkylsilyl derivatives, *viz.* N-triethylsilyl amino acid triethylsilyl esters, N-tripropylsilyl amino acid TMS esters, and N-tripropylsilyl amino acid tripropylsilyl esters. These were found to be no longer sufficiently volatile for general gas chromatographic purposes. RÜHLMANN AND MICHAEL also made some N-acetyl amino acid TMS esters and N-TFA amino acid TMS esters that were rapidly prepared and gave sharp symmetrical peaks on gas chromatography.

To obviate the interference due to MSA, lack of complete solubility of BSA in CH_3CN, and quantitation of silylation, GEHRKE and coworkers[11,12] in 1968 synthesized

a new chemical reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA). This new reagent was investigated as to the reaction conditions required for the precise and quantitative formation of the twenty TMS amino acid derivatives. It was found that BSTFA is an active silylating reagent, has increased volatility and appears with the solvent front, and has a lower detector response and greater solubility in some solvents than BSA. The fluorine in BSTFA results in less SiO_2 deposits and thus decreased detector noise. Silylation at 125° for 15 min in a closed tube resulted in single reproducible derivatives for eighteen of the twenty protein amino acids, and cystine was derivatized in 30 min at 150°. However, no reproducible chromatographic peak was obtained for arginine. Asp(NH_2) was converted to the TMS derivative in 30 min at 150°, and Glu(NH_2) in 30 min at 70°. BSTFA and its reaction product mono(trimethylsilyl)trifluoroacetamide (MSTFA) were found to be more volatile than BSA and did not interfere in the chromatographic separation of alanine and glycine. It was further observed that nonpolar liquid phases must be used in the chromatography of the TMS amino acid derivatives as decomposition occurs on polyester columns. From these studies it was concluded that silylation of amino acids and other biologically important molecules with BSTFA holds considerable promise as the derivatives can be prepared in a single step with little time.

A series of papers has just been published by GEHRKE and coworkers on the experimental conditions for silylation and GLC analysis of some biologically important groups of molecules: nucleic acid components[13,14], iodo-containing amino acids[15], sulfur-containing amino acids[16], and N-acetylneuraminic acid[17]. An extensive series of studies was made on the exact reaction conditions required for quantitative silylation of each organic class. Detailed methods are presented and data reported on the precision, accuracy, recovery, and application of the methods.

EXPERIMENTAL

In the following sections, the chemical derivatization method, chromatographic and instrumental conditions, and calculations are presented for the analysis of the TMS derivatives of the amino acids.

Reagents and materials

Acetonitrile. Obtained from Mallinckrodt Chemical Works, St. Louis; nanograde purity. Store over anhydrous $CaSO_4$ in a 1-liter screw top bottle with a teflon-lined cap.

Amino acids. Obtained from Mann Research Laboratories, New York, N.Y.; "Mann Assayed" chromatographically pure. Amino acids were also obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure.

Methylene chloride (anhydrous). Reflux 1000 ml of ACS reagent grade CH_2Cl_2 over 25 g of anhydrous $CaCl_2$ for 30 min. Distill in an all-glass apparatus and store in an all-glass inverted top bottle (Scientific Glass Apparatus Co., JB-1740) to protect from atmospheric moisture.

Bis(trimethylsilyl)trifluoroacetamide. Regis Chemical Co., Chicago. Store in a refrigerator at 4°.

Compressed gases. From Air Products and Chemicals, Inc., Kansas City, Mo. N_2, 99.995%; H_2, 99.0%; air, water pumped.

Bath oil. Fisher Scientific Thermally Stabilized Bath Oil No. 0-2.

Substrates. OV-7 (phenylmethyl 20/80 siloxane), and OV-22 (phenylmethyl 65/35 siloxane), Applied Science Laboratories, Inc., State College, Pa.

Support material. High-performance 100/120 mesh Chromosorb G. This support material was obtained from Johns-Manville Products Corporation, New York, N.Y.

Silanized spun glass wool. Analabs, Inc., North Haven, Conn. Store in a desiccator over P_2O_5.

Stock solutions

Standard amino acids solution. An aqueous solution (0.1 *N* HCl) containing the twenty protein amino acids at individual concentrations as given in Table I.

Phenanthrene internal standard solution. An acetonitrile solution of phenanthrene, 1.023 mg/ml (100 ml), 5.74×10^{-3} mmole/ml.

TABLE I

GRAMS OF AMINO ACIDS REQUIRED FOR A 2.5 MILLIMOLAR STOCK SOLUTION

Molecular weights of the free amino acids are given. These values must be altered when the amino acid hydrochlorides are used.

Amino acid	*Molecular weight (g)*	*Amount required*	
		g	*μg/10 μl*
Alanine	89.1	0.2228	2.228
Glycine	75.1	0.1878	1.878
Valine	117.1	0.2928	2.928
Leucine	131.2	0.3280	3.280
Isoleucine	131.2	0.3280	3.280
Proline	115.1	0.2878	2.878
Serine	105.1	0.2628	2.628
Threonine	119.1	0.2978	2.978
Hydroxyproline	131.1	0.3278	3.278
Aspartic acid	133.1	0.3328	3.328
Methionine	149.2	0.3730	3.730
Cysteine	121.2	0.3030	3.030
Glutamic acid	147.1	0.3678	3.678
Phenylalanine	165.2	0.4130	4.130
Arginine	174.2	0.4355	4.355
Lysine	146.2	0.3655	3.655
Tyrosine	181.2	0.4530	4.530
Histidine	155.2	0.3880	3.880
Tryptophan	204.2	0.5105	5.105
Cystine	240.3	0.6008	6.008

Apparatus and glassware (macro and semimicro method)

The oil bath, in which the closed-tube trimethylsilylation reaction was conducted, consisted of a $3\frac{1}{2}$ in. $\times$ 4 in. $\times$ 6 in. aluminum pan supported on a hot plate to maintain uniform temperature of the oil bath. Temperature control was achieved with two 100-W heaters, and a Variac. A sand bath can be used.

A super D-21-36 safety shield obtained from Instruments for Research and Industry (I^2R) was used to provide protection from accidental breakage of the silylation reaction vessel.

An all-teflon rotary evaporator obtained from California Laboratory Equipment

Company (Calif. Lab. Model C rotary evaporator) was used to remove the volatile solvents. The vacuum was produced with a Welch Duo-Seal vacuum pump (W. M. Welch Scientific Co., Chicago, Ill.) having a capacity of 140 l/min. 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. A sodium hydroxide trap was placed between the condenser and the vacuum pump to protect it from corrosive acidic compounds.

Silylation reaction tube. A standard pyrex glass, Corning Glass Works Co. No. 9826, 16 × 75 mm, screw-cap culture tube with teflon-lined cap was used.

Dry heated bath. Thermolyne Corporation, Dubuque, Iowa. Constant temperature 106°.

Syringes. Hamilton 701 N, 10 μl, Hamilton Co., Whittier, Calif.

Filter-driers for the carrier gas of the gas chromatograph were obtained from MicroTek Instruments, Inc., Baton Rouge, La. (Catalog No. 830041), and contained activated carbon, silica gel, and $CaSO_4$. Filters for the N_2 gas used in evaporating the solvents from the samples were obtained from The Koby Corp., Melrose, Mass. (Catalog No. 93975) and contained activated charcoal and $CaSO_4$. Filter-driers can also be prepared by packing 10-in. by 1-in. metal cylinders with silica gel and Linde molecular sieve type 5A. Filters are recommended for the nitrogen, hydrogen, and air lines to remove water and hydrocarbons.

Instrumental and chromatographic conditions

Minimum. Single-column instrument with temperature programming, single hydrogen flame-ionization detector, and recorder equipped with disc integrator. Instrument should have glass injection ports or glass injector liners which can be inspected for buildup of deposits and readily replaced and cleaned. Early experiments indicated that threonine and arginine were decomposed when injected into a hot metal flash heater. The use of direct on-column injection eliminated problems of derivative thermal breakdown. When glass injectors were used in metal columns at elevated temperatures, no adverse effects on these or other amino acid derivatives were observed. The availability of multiple program rates greatly facilitates the chromatographic examination of samples, since the program rate may be systematically varied. The resolution of derivatives with high retention temperatures is more readily achieved using a more rapid program rate (4 to 6°/min) than the resolution of derivatives with low retention temperatures.

Instrumental operation and chromatographic conditions used

Instruments used. MicroTek Model MT-220 automatic sequential programmed-temperature instrument equipped with a four-column oven bath, with two dual-flame ionization detectors, Model 73980, and two electrometers, Model 636800. It was also equipped with a linear temperature programmer and a Varian Model 20 dual pen recorder. Each of the four columns could be operated independently. A Packard Instruments Co. Model 7300 dual column gas chromatograph with two flame ionization detectors and equipped with a Honeywell Electronic 16 strip chart recorder was also used. The chromatographs were equipped with an Infotronics magnetic tape recorder Model CRS-42 RSI and a digital readout system Model CRS-11AB/HS/42, and a CRS-104 digital integrator.

Conditions. The chromatographic conditions used with the columns of the mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 on high-performance (H.P.) 100/120 mesh Chromosorb G in a 1.75 m × 4 mm I.D. U-shaped borosilicate glass column were:

Column temperature:	initial 75 and 100°, final 225°
Program rate:	2°/min
Detector temperature:	230°
Sensitivity: macro	10 × 64
semimicro	10 × 16
Carrier flow, N_2:	42 ml/min
Air (to detector):	475 ml/min
Hydrogen (to detector):	30 ml/min
Chart speed:	0.33 in./min

Substrates and supports. A mixed liquid phase of OV-7 (20/80% phenylmethylsiloxane) and OV-22 (65/35% phenylmethylsiloxane) was used for the separation of the TMS derivatives of the amino acids. Acid-washed (a.w.) H.P. Chromosorb G 100/120 mesh was used with the mixed liquid phase. The liquid phase was coated on the a.w. H.P. Chromosorb G at a concentration of 3.0 w/w% OV-7, and 1.5 w/w% OV-22, then packed in a 1.75 m × 4 mm I.D. glass column.

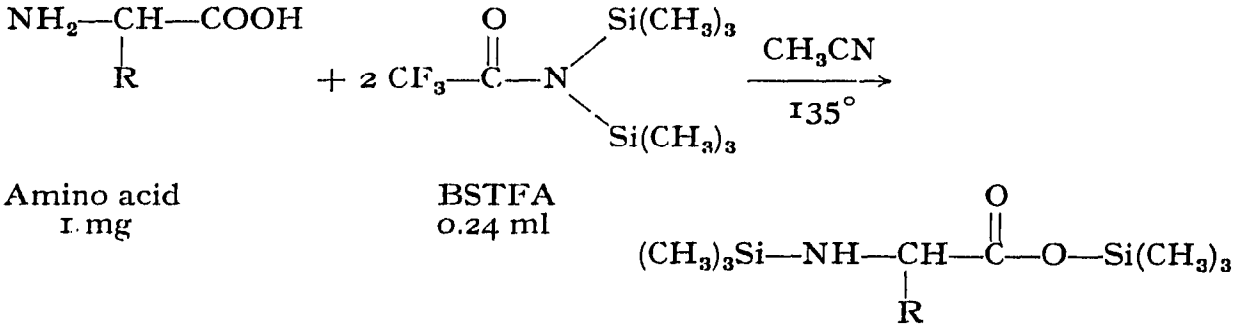
Column preparation. To prepare 30.00 g of column packing, weigh 0.90 g of OV-7 into a 50-ml Erlenmeyer flask, and weigh 0.45 g of OV-22 into another Erlenmeyer flask. Dissolve each with anhydrous methylene chloride (25 to 30 ml), respectively. Weigh 28.65 g of 100/120 mesh a.w. H. P. Chromosorb G into a 500-ml ribbed round bottom flask. Add methylene chloride until the liquid level is $\frac{1}{4}$ in. above the Chromosorb G. Then quantitatively transfer the OV-7 and OV-22 solutions to the flask containing the Chromosorb G and methylene chloride. Evaporate the slurry to dryness, *slowly* to achieve uniform coating with a rotary evaporator and a 60° water bath. (30 g of packing is sufficient to pack two 1.75 m × 4 mm I.D. columns.) The column material is now ready for packing in clean, dry glass columns. The column should be gently tapped during filling with the packing to ensure uniform distribution. A plug of silanized spun glass wool is then packed in each end of the column to hold the column packing in place. After placing the column in the oven bath, it is conditioned for at least 24 h at 235° ± 5° with a carrier flow rate of 30 to 35 ml/min of N_2 gas.

A properly prepared column gives excellent separation for at least three months, however, it is recommended that a performance standard amino acid mixture be analyzed periodically to evaluate any change in the separation characteristics of the column. *Note.* It is essential that moisture be excluded from the support materials, liquid phases, and solvents during the total preparation procedure.

Derivatization method—preparation of TMS amino acids

General. The chemical derivatization of amino acids involves the following steps: (1) Removal of water to give the dry amino acid hydrochlorides. (2) Trimethylsilylation of the amino acids.

Trimethylsilylation of an amino acid



The reaction is conducted in a closed tube at 135° for 15 minutes *or* 4.0 h. The BSTFA/CH_3CN ratio is 1:1.

Analytical derivatization methods

Macro and semimicro method (20 mg to 100 μg)

(1) Add an aqueous aliquot containing 0.1–20.0 mg of total amino acids to a No. 9826 culture tube (Note 1).

Evaporate the solution *just to dryness* by placing the tube in a dry, heated sand bath at 106° while directing a regulated stream of *filtered*, dry N_2 gas (*ca.* 100 ml/min) into the heated tube, or place tube on a black surface under an IR lamp.

(3) Add 0.5 ml of CH_2Cl_2 and evaporate as in Step 2 to ensure complete azeotropic removal of water. *Repeat*, take *just to dryness*.

(4) Add an appropriate *exact* amount of phenanthrene internal standard solution (1–0.1 mg) or an amount equivalent to one of the amino acids in higher concentration.

(5) Add 30 molar excess BSTFA (0.24 ml of BSTFA per 1 mg of amino acids) (Note 2), and the same volume acetonitrile, close tube securely with a teflon-lined cap, and effect solution by manual inversion and ultrasonic mixing.

(6) Trimethylsilylate at 135° in an oil or sand bath for 15 min. Remove the reaction tube after 15 min and *cool immediately* under the tap, dry with tissue paper.

Chromatograph 3–10 μl in the gas chromatograph equipped with a 1.75 m × 4 mm I.D. glass column containing 100/120 mesh a.w. H.P. Chromosorb G which has been coated with a mixture of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 and properly conditioned. After an initial hold for 7 min, program the oven temperature from 75° at 2°/min (Note 3). The detector temperature should be maintained at 255° and the injection port at 300°. The carrier gas (N_2) flow rate should be adjusted to achieve maximum separation. This corresponds to a flow rate of 42 ml/min with a pressure of 40 p.s.i.g.

(7) During chromatography, re-silylate the same reaction tube at 135° for 4 h (Note 4). This additional reaction time is necessary for six of the amino acids, *viz.* GLU, ARG, LYS, HIS, TRY, and CYS.

(8) Chromatograph 3–10 μl of the sample (Step 7) on the same column. After an initial hold for 7 min, program the oven temperature from 100° at 2°/min (Note 5).

Note 1. All glassware must be thoroughly washed, rinsed repeatedly with double distilled water, and dried.

Note 2. Various volumes of BSTFA and acetonitrile were investigated for the complete silylation of 1 mg of amino acid. Also, experiments were made with BSTFA alone, without solvent added. A critical evaluation of all the data showed that the best results for the complete derivatization of the twenty natural protein amino acids was obtained with a solution of 0.24 ml of BSTFA and 0.24 ml of acetonitrile for 1 mg of amino acid.

Note 3. About 80 min were required to obtain a total chromatogram from the first peak to the last, cystine.

Note 4. After the initial injection (Step 6), the reaction tube was securely closed and again placed in the oil bath and heated at 135° for 4 h. A reaction temperature of 135° and time of 15 min gave three small peaks for arginine. These are the 2-TMS, 3-TMS, and 4-TMS derivatives (ARG_2, ARG_3, ARG_4). Lysine gave a 3-TMS and a 4-TMS derivative (LYS_3, LYS_4). Both the 2-TMS and 3-TMS derivatives were obtained for histidine and tryptophan, respectively (HIS_2, TRY_3). A large 3-TMS derivative was obtained for glutamic acid. However, a small amount of cyclized compound was also obtained for glutamic acid, and is the 2-pyrrolidone-5-carboxylic acid TMS derivative. Cystine gave a small peak. However, when the reaction conditions were changed to 135° for 4 h, arginine, lysine, and histidine were completely converted to the trimethylsilylated derivative, and each gave one peak. Under these conditions, tryptophan gave a large 3-TMS peak and a much smaller 2-TMS peak. The RMR of TRY_2/TRY_3 was constant with a value of 0.18, and the RMR of GLU_2/GLU_3 was 4.15, and remained constant. Glutamic acid was converted mainly to the 2-pyrrolidone-5-carboxylic acid TMS derivative, with a small amount of the 3-TMS glutamic acid derivative remaining. Many different experimental conditions of temperature and time were investigated. The temperatures studied were: 125°, 135°, 150°, and 170°; reaction times varied from 15 min to 13 h. An evaluation of all the different experimental conditions showed that 135° for 4 h was the best set of conditions.

Note 5. A complete chromatogram required about 60 min from the first peak to the last peak, cystine.

Internal standard method of calculation

This method was used to calculate the mole%, or w/w%, for each amino acid. The calculation of the absolute amounts of amino acid in a sample is best accomplished by the use of an internal standard (I.S.). Phenanthrene was found suitable as an internal standard because it was well resolved from the amino acids on a column of the mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 on 100/120 mesh H.P. Chromosorb G.

$$RMR_{a.a./phen} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$$

In these experiments, electronic and disc integration were used and peak areas were measured as counts. After the molar response of each amino acid has been determined relative to an I.S. (phenanthrene) from at least three independent analyses, these values may then be used to calculate the quantity of amino acids present in a sample.

$$\mathrm{RMR_{a.a./I.S.}} = \frac{\dfrac{A_{a.a.}}{\dfrac{g_{a.a.}}{\mathrm{GMW_{a.a.}}}}}{\dfrac{A_{I.S.}}{\dfrac{g_{I.S.}}{\mathrm{GMW_{I.S.}}}}}$$

After the addition of an *exact known amount* of the I.S. to the sample and subsequent analysis by GLC, the following formula may be used to calculate the amount of each amino acid present in the sample.

$$g_{a.a.} = \frac{A_{a.a.} \times \mathrm{GMW_{a.a.}} \times g_{I.S.}}{A_{I.S.} \times \mathrm{GMW_{I.S.}} \times \mathrm{RMR_{a.a./I.S.}}}$$

$$\text{w/w \% of amino acid} = \frac{\text{grams of amino acid}}{\text{grams sample}} \cdot 100$$

GMW denotes gram molecular weight of amino acid.

Comments on internal standard method

(1) Gives exact mole%, or w/w%, of an amino acid with a minimum of instrumental calibration.

(2) Does not require the preparation of calibration curves for amino acids. This requires considerable work if pure reference standards are not available.

(3) Does not require a calibration curve for the internal standard.

(4) Does require information on the *exact amount* of internal standard added to the sample flask.

(5) Does require information on the relationship of $\mathrm{RMR_{a.a./I.S.}}$

(6) Dilution or concentration of sample, after addition of internal standard are of little importance. The area ratio for the unknown and the I.S. remains constant, providing there is no selective substrate–amino acid derivative interaction, or selective loss of I.S. or derivative in some other way.

(7) Amount of derivatized sample injected is not critical as long as a well-sized peak is obtained.

The proposed structures of the N-trimethylsilyl TMS esters of the twenty protein amino acids are given in Table II. Further positive identification of the derivatives will necessitate elemental analysis and other instrumental studies on the pure synthesized derivatives.

RESULTS AND DISCUSSION

Chromatographic separation studies

An evaluation was made of various OV (phenylmethylsiloxane) substrates and combinations for the separation of the twenty protein amino acids. Preliminary experiments with various substrate loadings indicated that difficulties would be encountered in achieving a complete separation.

A fairly acceptable separation was achieved with a 3.0 w/w% 2.0 m column of OV-7 on 100/120 mesh H.P. Chromosorb G (Fig. 1). An extensive series of experiments was then conducted in which the experimental conditions of initial temperature, iso-

thermal hold time, carrier gas flow rate, temperature programming, and column length were evaluated. These experiments were made over a period of about three months, and it was noted that changes in these parameters significantly affected the separation. From this work the best experimental conditions were defined, and these are described in the section EXPERIMENTAL under *Conditions*. For the samples that were derivatized at 135° for 15 min, the initial column temperature was 75°; and for

TABLE II

STRUCTURE OF THE TMS DERIVATIVES OF AMINO ACIDS

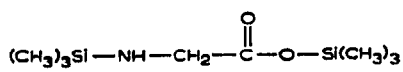
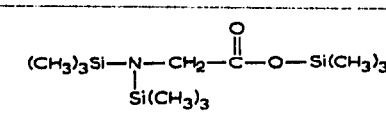
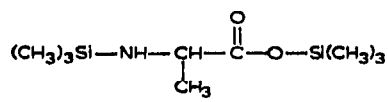
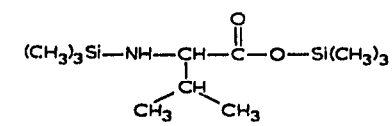
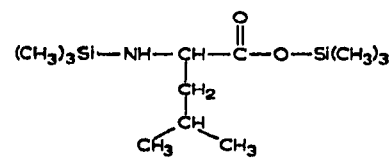
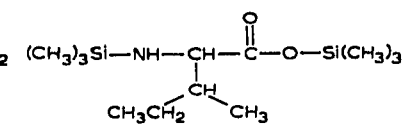
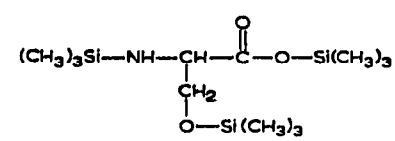
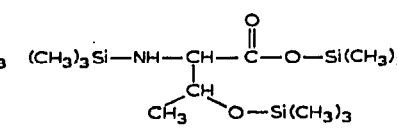
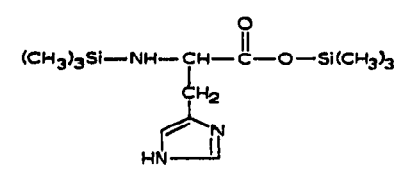
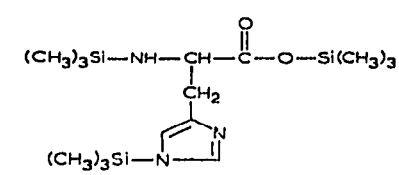
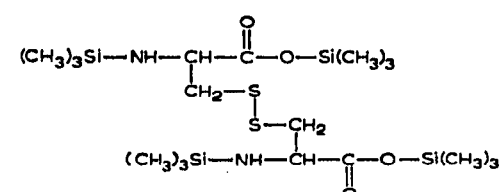
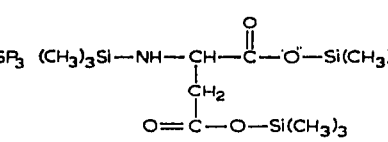
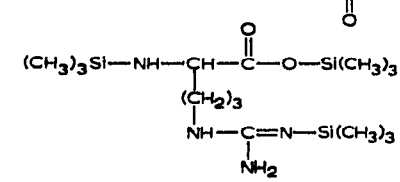
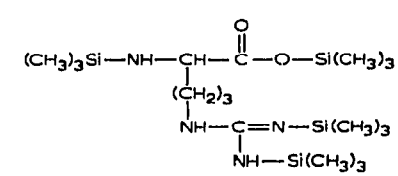
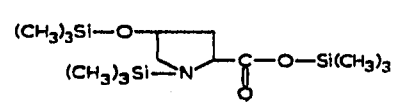
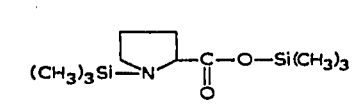
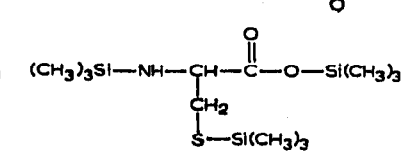
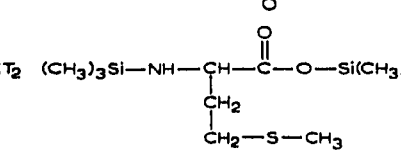
Glycine, GLY_2		Glycine, GLY_3	
Alanine, ALA_2		Valine, VAL_2	
Leucine, LEU_2		Isoleucine $ILEU_2$	
Serine, SER_3		Threonine, THR_3	
Histidine, HIS_2		Histidine, HIS_3	
Cystine, CYS_4		Aspartic acid, ASP_3	
Arginine, ARG_3		Arginine, ARG_4	
Hydroxyproline, $HyPRO_3$		Proline, PRO_2	
Cysteine, $CYSH_3$		Methionine, MET_2	

TABLE II *(continued)*

Lysine, LYS_3	$(CH_3)_3Si-NH-CH(-(CH_2)_4-NH-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$	Lysine, LYS_4	$(CH_3)_3Si-NH-CH(-(CH_2)_4-N(-Si(CH_3)_3)-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$
Tyrosine, TYR_3	$(CH_3)_3Si-NH-CH(-CH_2-C_6H_4-O-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$	Phenylalanine, PHE_2	$(CH_3)_3Si-NH-CH(-CH_2-C_6H_5)-C(=O)-O-Si(CH_3)_3$
Tryptophan, TRY_2	$(CH_3)_3Si-NH-CH(-CH_2-C_8H_6N)-C(=O)-O-Si(CH_3)_3$	Tryptophan, TRY_3	$(CH_3)_3Si-NH-CH(-CH_2-C_8H_6N-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$
Glutamic acid, GLU_3	$(CH_3)_3Si-NH-CH(-(CH_2)_2-C(=O)-O-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$	2-Pyrrolidone-5-carboxylic acid, GLU_2	$(CH_3)_3Si-N(-CH(-CH_2-CH_2-C(=O))-C(=O)-O-Si(CH_3)_3)-C(=O)$

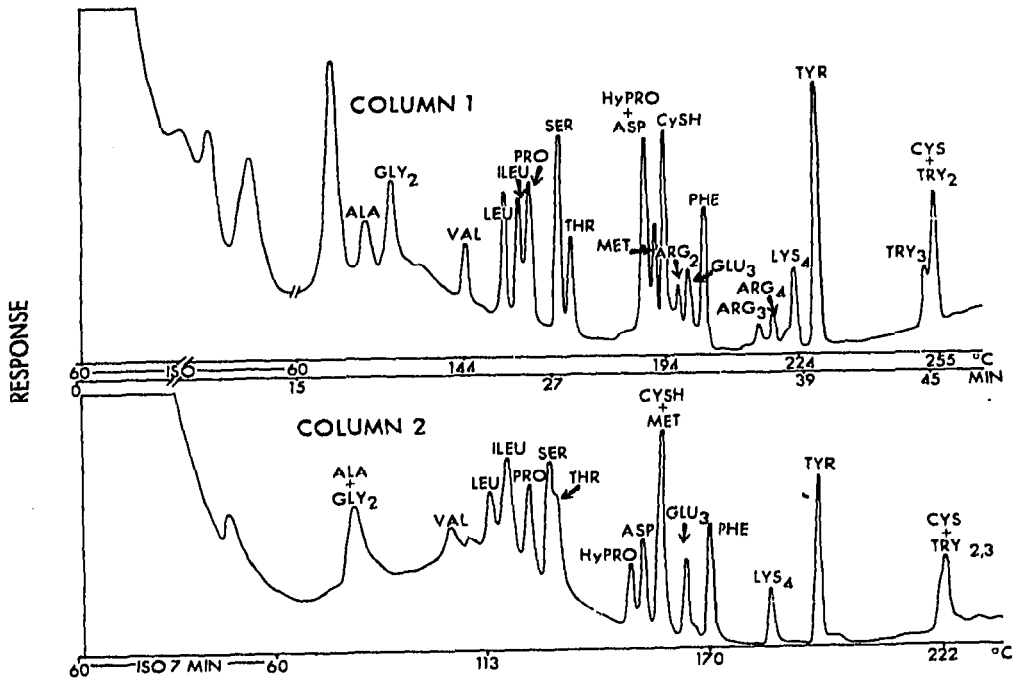


Fig. 1. Separation of TMS amino acids. Column 1: 3.0 w/w % OV-7 on 100/120 mesh H.P. Chromosorb G, 2.0 m × 4 mm I.D., glass; initial temperature 60°, 15 min hold, then 7.5°/min. Column 2: 1.5 w/w % OV-22 on 100/120 mesh H.P. Chromosorb G, 2.0 m × 4 mm I.D., glass; initial temperature, 60°, 7 min hold, then 7.5°/min.

the samples derivatized at 135° for 4 h, 100°. In each case the temperature was programmed to a final temperature of 225°.

Eighteen of the amino acids were separated on a 4.0 w/w%, 1.75 m × 4 mm I.D., column of OV-7 coated on a.w. 100/120 mesh H.P. Chromosorb G. Hydroxyproline and aspartic acid were the only two amino acids that overlapped. The separation of these two TMS amino acid derivatives was achieved on a 1.5 w/w%, 1.75 m × 4 mm I.D., column of OV-22 on a.w. 100/120 mesh H.P. Chromosorb G. However, with this column four other pairs of amino acids had the same retention temperatures. These were alanine and glycine, serine and threonine, methionine and cysteine, and tryptophan and cystine (Fig. 1). From information gained in these experiments a mixed liquid phase of OV-7 and OV-22 was next tried. This resulted in the complete separation of the TMS derivatives of the twenty protein amino acids with phenanthrene as I.S. on a *mixed* liquid phase of 3.0 w/w% OV-7 plus 1.5 w/w% OV-22 on an a.w. 100/120 mesh H.P. Chromosorb G column, 1.75 m × 4 mm I.D.

Sixteen of the amino acids were soluble in BSTFA. Aspartic and glutamic acids, cystine, and histidine were only partially soluble. Thus it was necessary to use a polar solvent with BSTFA in the derivatization reaction. Acetone, tetrahydrofuran, dioxane, and acetonitrile were investigated. The best solvent was acetonitrile. All of the amino acids were soluble with a BSTFA/CH_3CN ratio of 1:1.

In chromatographic experiments it was further found that H.P. Chromosorb G was necessary to obviate the tailing of the acetonitrile solvent peak into alanine and glycine as these amino acids were eluted from the column. Acid-washed Chromosorb G was not satisfactory as a support phase. The silanized H.P. Chromosorb G was entirely satisfactory and apparently all of the hydroxyl groups were masked and thus interaction of the solvent with the support was decreased. With the above recommended *mixed* liquid phase and support material, the life of a column was found to be at least three months. Figs. 2 and 3 represent typical chromatograms for the GLC separation

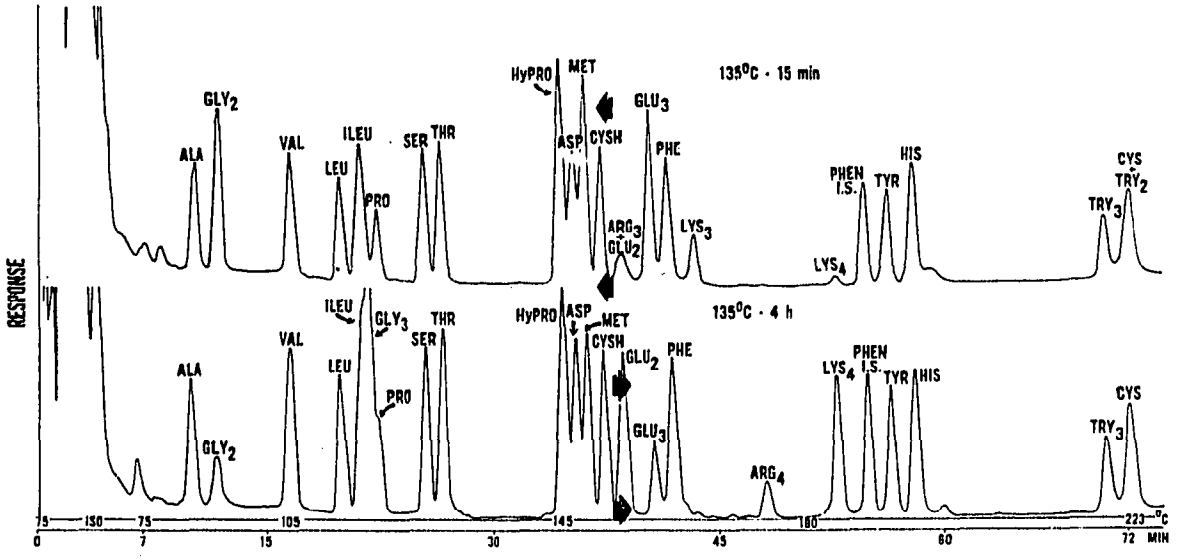


Fig. 2. GLC chromatogram of TMS protein amino acids. Sample: 13.06 mg in 6.2 ml, 21.06 μg total amino acids injected (10 μl). BSTFA/CH_3CN ratio 1:1. Column: mixed liquid phase of 3.0 w/w % OV-7 and 1.5 w/w % OV-22 on 100/120 mesh H.P. Chromosorb G, 1.75 m × 4 mm I.D., glass. Initial temperature 75°, 7 min hold, then 2°/min.

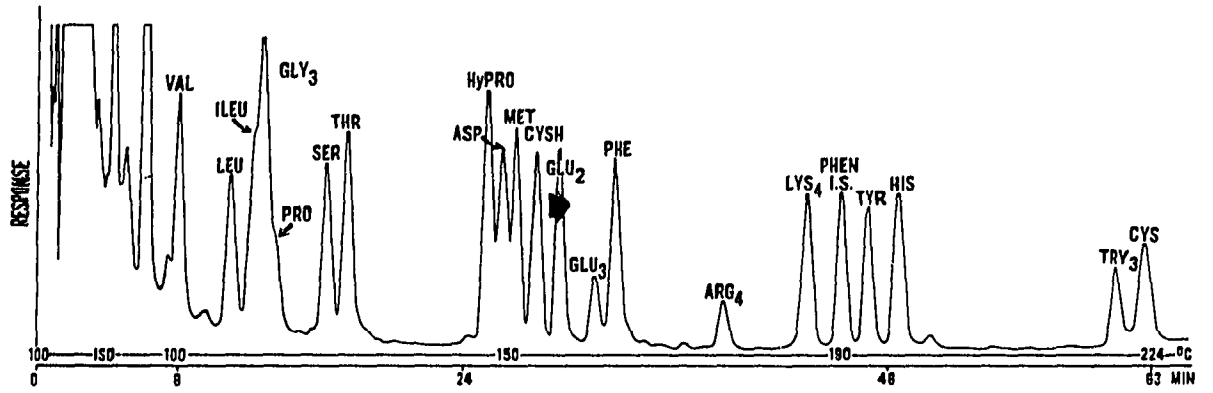


Fig. 3. GLC chromatogram of TMS protein amino acids; 135° for 4 h. Sample: 13.06 mg in 6.2 ml, 21.06 μg total amino acids injected (10 μl). BSTFA/CH_3CN ratio 1:1. Initial temperature 100°, 8 min hold, then 2°/min. Column, same as Fig. 2.

of the TMS protein amino acids. Details for the derivatization, instrumental, and chromatographic conditions are given in the legends to the figures. Fourteen of the amino acids, including phenylalanine and tyrosine (Fig. 2, top), must be derivatized at 135° for 15 min; and for six of the amino acids (GLU_2, ARG_4, LYS_4, HIS_3, TRY_3, and CYS_4) the required derivatization temperature and time were 135° for 4 h (Fig. 2, bottom).

Injection port temperature. For the chromatography of the TMS amino acids it was found necessary to maintain the injection port temperature at 250–300°. At lower temperatures incomplete elution of the less volatile derivatives (HIS_3, TRY_2, TRY_3, and CYS_4) occurred, resulting in reduced responses and reappearance of these peaks as ghosts or breakdown products in subsequent chromatographic analysis.

Solvent tailing. In some instances it was noted that the solvent peak of the reaction mixture (BSTFA, MSTFA, and CH_3CN) tailed badly, thus interfering with the quantitation of the most volatile TMS amino acid derivatives (ALA_2, GLY_2, and VAL_2).

Tailing of the solvent peak was apparently a result of the loss of the liquid phase in that portion of the column which was in direct contact with the hot injection port, maintained at 250–300°. The solvent was probably adsorbed on the surface of the altered packing material, and thus was less rapidly eluted than would be the case if the support were coated with liquid phase. This problem can be obviated by removing the packing from that portion of the column which comes in direct contact with the heated injection port.

Column conditions. Some important observations and points of emphasis about the chromatographic column and the separations are: the exact w/w% of the OV-7 and OV-22 liquid phases used are not too critical. One of the first observations that a chromatographic column is deteriorating was indicated by the many small peaks between HIS_3 and CYS_4, and resolution was lost first for phenanthrene (I.S.), TYR_3, and HIS_3. Other indications were that the base line between BSTFA and ALA_2 was not sharp, the CH_3CN solvent peak tailed into the ALA_2 peak, and the ALA_2 peak appeared early. To achieve a good separation of the twenty amino acids the flow rate of carrier gas (N_2) is most critical (40–42 ml/min), and next the temperature program rate (2°/min).

Performance blank

Performance blank studies were made on all reagents, and with the chromatographic, and instrumental conditions as presented to determine the existence and source of extraneous peaks and contamination. Chromatograms were made for acetonitrile, BSTFA, BSTFA heated at 135° for 15 min, BSTFA plus CH_3CN heated at 135° for 15 min, and BSTFA heated at 135° for 4 h. A good blank chromatogram was achieved for CH_3CN at the sensitivity settings normally used. However, the commercial BSTFA sometimes showed extraneous peaks. The retention temperatures of these extraneous peaks were at 100°, 115°, and 140°, and a careful distillation removed much of the extraneous material. When the BSTFA was heated at 135° for 15 min, these extraneous peaks became larger; at 135° for 4 h, these three peaks were quite large and other peaks appeared. All of them were found below a retention temperature of 145°. It was most fortunate that these extraneous peaks did not occur at the same retention temperatures for any of the protein amino acids. The commercial BSTFA that is now available does not show these extraneous peaks. In general, these peaks resulted from the silylation of trace impurities present in BSTFA, or from break-down products of impure BSTFA which were then silylated. It is essential that only highly purified BSTFA should be used.

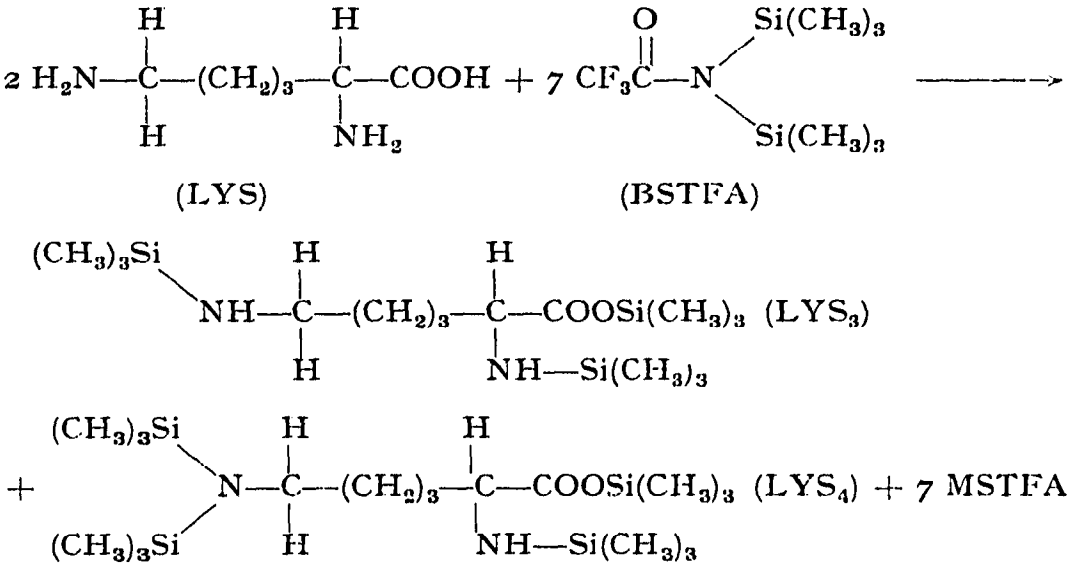
Derivatization reaction conditions

Some derivatization experiments were conducted using only BSTFA and no organic solvent. Other experiments were conducted using solvents of different polarities to help dissolve four of the amino acids. Acetonitrile was found to be the best solvent at an equal volume to BSTFA. The following reaction temperatures were investigated: 125°, 135°, 150°, and 170°. Reaction times were: 5, 10, 15, and 30 min, and 1, 2, 3, 4, 5, 6, and 13 h. The molar excess of BSTFA to the total amino acids studied was: 8, 10, 20, 30, 50, and 100. The following amino acids gave multiple peaks under different experimental conditions: glycine, glutamic acid, arginine, lysine, histidine, and tryptophan. Multiple peak formation for arginine and lysine occurs because these amino acids have an ω-amino group which is silylated to different extents; histidine and tryptophan have a heterocyclic nitrogen atom; glutamic acid gives a cyclized compound 2-pyrrolidone-5-carboxylic acid TMS derivative; and the α-amino group on glycine can be doubly silylated due to lack of steric hindrance.

Trimethylsilylation reaction for lysine

For all the amino acids except glycine, the α-amino group is substituted by only one TMS group, whereas the ω-amino group is substituted by one or two TMS groups, depending upon the reaction temperature and time. This is due to the presence of steric hindrance to the α-amino group for all of the amino acids except glycine.

The best reaction conditions for the trimethylsilylation of fourteen amino acids is at 135° for 15 min. Phenylalanine and tyrosine are included in the fourteen. Refer to the top chromatogram in Fig. 2. However, to quantitatively convert the remaining six amino acids, it was necessary to heat at 135° for 4 h. These amino acids are GLU_2, ARG_4, LYS_4, HIS_3, TRY_3, and CYS_4. When glycine was derivatized at 135° for 15 min, the ratio of GLY_2/GLY_3 was found to be 6.7 and remained constant. Trimethylsilylation at 135° for 4 h gave a ratio of 0.10 for GLY_2/GLY_3. Glycine is included with the fourteen amino acids because GLY_3 has a retention temperature between that for



isoleucine and proline, and thus interfered in the separation of these two amino acid derivatives (Figs. 2 and 3).

To trimethylsilylate and to chromatographically separate the twenty amino acids, two sets of reaction conditions and two injections were required. The sample was initially heated at 135° for 15 min, then an aliquot was injected onto the column at an initial temperature of 75°. The reaction vessel was closed and again heated at 135° for 4 h, after which time a second injection of the sample was made, with the initial temperature of the column at 100°. After the first injection the column was programmed to about 225° and then cooled to the initial holding temperature of 100° prior to the second injection. An isothermal hold was also used at the beginning of each chromatogram. The amino acids from alanine to cysteine are included in the first chromatogram, plus phenylalanine and tyrosine; and from glutamic acid to cystine in the second chromatogram. Glutamic acid was converted into a cyclized compound 2-pyrrolidone-5-carboxylic acid TMS derivative (GLU_2, Table II). At the reaction conditions of 135° for 15 min, GLU_2 gave only a small peak; whereas at 135° for 4 h the pyrrolidone derivative was the major peak, and the remaining GLU_3 did not interfere in the chromatography. The glutamic acid content of the samples was determined by the summation of the areas of GLU_2 and GLU_3.

The trimethylsilylation of a number of the amino acids as a function of reaction conditions is presented in Fig. 4. One peak was obtained for ARG_4, LYS_4, HIS_3, and CYS_4, respectively. At 135° for 4 h, tryptophan yielded two peaks, TRY_3 and TRY_2 (Table II). The RMR ratio for TRY_2/TRY_3 was found to be 0.18 and remained constant. The GLC chromatogram of the TMS derivatives of tryptophan and cystine is shown in Fig. 5. Although TRY_2 and CYS_4 have the same retention temperature, the CYS_4 content can be measured by subtracting 18% of the total area of TRY_3 from the total area for TRY_2 plus CYS_4 (Fig. 5). From the above experiments on trimethylsilylation conditions, studies on the relative molar response of the amino acid derivatives as a function of temperature (Table III), and molar excess of BSTFA (Table IV), it was concluded that the best reaction conditions for the twenty amino acids were as follows: Take 1 mg total amino acids, add 0.24 ml BSTFA and 0.24 ml CH_3CN.

TABLE III

RELATIVE MOLAR RESPONSE OF TMS AMINO ACIDS AS A FUNCTION OF REACTION TEMPERATURE

Molar ratio of BSTFA/Total amino acids = 30. BSTFA/CH_3CN ratio, 1:1. Each value is an average of at least two independent analyses.

Amino acid	*$RMR_{a.a./phen.}$ and silylation conditions*			
	125°—15 min	*135°—15 min*	*135°—4 h*	*150°—2 h*
ALA_2	0.35	0.53	0.32	—
GLY_2	0.46	0.48	0.00	0.00
GLY_3	0.08	0.09	0.46	0.44
VAL_2	0.68	0.87	0.51	—
LEU_2	0.78	0.94	0.88	—
$ILEU_2$	0.95	1.06	1.04	—
PRO_2	0.43	0.61	0.36	—
SER_3	0.87	0.96	1.00	—
THR_3	0.88	1.06	0.96	—
$HyPRO_3$	0.93	1.14	0.96	—
ASP_3	0.84	0.86	0.80	0.66
MET_2	0.48	0.56	0.52	—
$CYSH_3$	0.45	0.52	0.21	0.27
GLU_2	0.13	0.15	0.54	—
GLU_3	0.52	0.53	0.15	—
PHE_2	1.02	1.08	0.93	0.85
ARG_4	—	—	0.51	0.24
LYS_4	—	—	0.89	0.52
TYR_3	1.36	1.53	1.44	—
HIS_3	—	—	0.55	0.29
TRY_3	—	—	0.87	—
TRY_2	—	—	0.16	—
CYS_4	—	—	0.81	0.98

TABLE IV

RELATIVE MOLAR RESPONSE OF TMS AMINO ACIDS AS A FUNCTION OF MOLAR RATIO OF BSTFA

Closed tube silylation at 135—°15 min. BSTFA/CH_3CN ratio, 1:1. Molar ratio of BSTFA/Total amino acids = 20, 30 and 50. Each value is an average of at least two independent analyses.

Amino acid	*$RMR_{a.a./phen.}$ and molar ratio*		
	20	*30*	*50*
ALA_2	0.41	0.53	0.54
GLY_2	0.49	0.48	0.36
GLY_3	0.11	0.09	0.18
VAL_2	0.88	0.87	0.86
LEU_2	0.96	0.94	0.90
$ILEU_2$	0.97	1.06	1.12
PRO_2	0.58	0.61	0.58
SER_3	0.93	0.96	0.86
THR_3	1.00	1.06	0.92
$HyPRO_3$	1.15	1.14	0.99
ASP_3	0.85	0.87	0.83
MET_2	0.78	0.77	0.73
$CYSH_3$	0.54	0.54	0.42
PHE_2	1.02	1.08	—
TYR_3	1.36	1.53	1.56

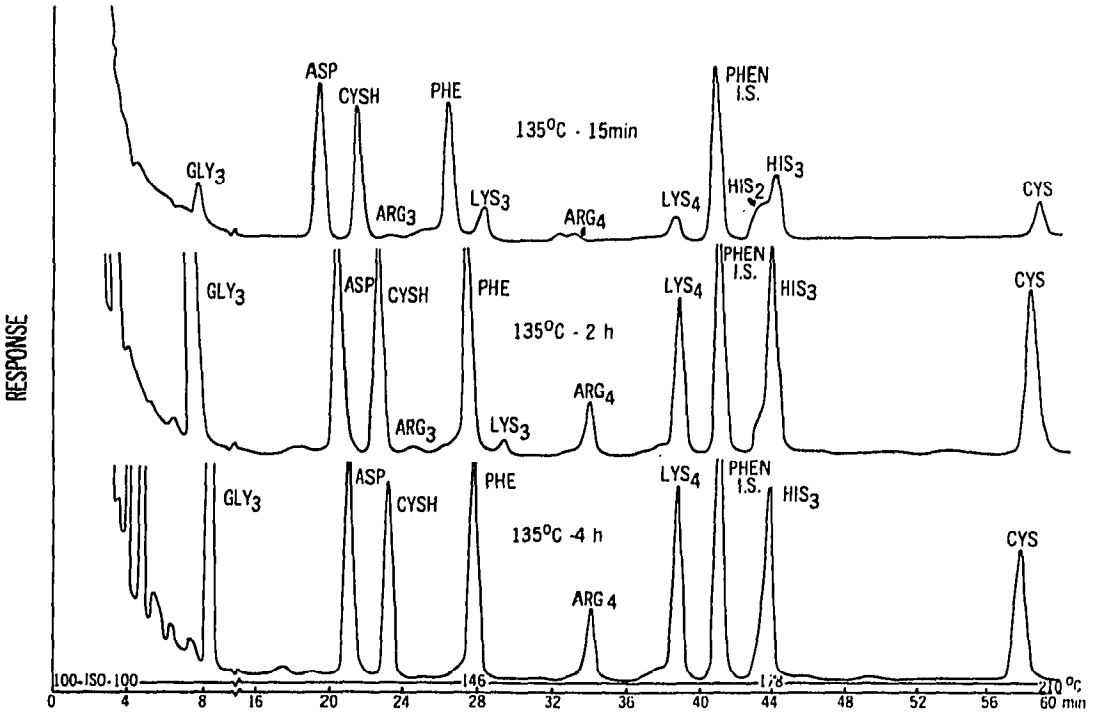


Fig. 4. Silylation of amino acids as a function of reaction time. Sample: 6.3 mg in 4.8 ml, 10.5 μg total amino acids injected (8 μl). BSTFA/CH_3CN ratio 1:1. Initial temperature 100°, 4 min hold, then 2°/min. Column, same as Fig. 2.

Heat at 135° ± 2° for 15 min ± 1 min, followed by heating at 135° ± 2° for 4 h ± 10 min.

Relative molar response of TMS amino acids

The relative molar response values, $RMR_{a.a./phen.}$, for fourteen of the TMS amino acid derivatives silylated under the optimum conditions of 135° for 15 min are given in Table V. The RMR values for the remaining six amino acids silylated at 135° for 4 h are presented in Table VI. The number of TMS groups attached to each amino acid is denoted as a subscript. Phenanthrene was used as I.S. and was arbitrarily assigned a value of unity. Each solution contained *ca.* 0.5 mg of each amino acid and 0.5 mg of I.S. The amino acids were analyzed singly as well as in groups, and no interactions were observed. All of the chromatographic experiments were made on the mixed liquid phase of OV-7 and OV-22 at a w/w% ratio of 3/1.5. No amino acid derivative–substrate interactions were noted with the siloxane substrate phases. However, it was earlier noted that polyester substrates could not be used for chromatography of the TMS derivatives. Under the experimental conditions used, the RMR values were very reproducible; and the average RSD (%) for the fourteen amino acids (Table V) was found to be 0.80, and the average RSD (%) for the remaining six amino acids (Table VI) was found to be 1.25. It was noted in general that the RMR values for the fourteen amino acids decreased when the silylation time was increased to 4 h (Table V). Data are presented in Table VII giving the RMR values for a number of the TMS amino acid derivatives as a function of reaction time. It was observed that the optimum silylation time for GLU_2, ARG_4, LYS_4, HIS_3, TRY_3, and CYS_4 was 4 h.

TABLE V

RELATIVE MOLAR RESPONSE OF THE TMS AMINO ACIDS

Optimum conditions, 135°—15 min. The number of TMS groups attached to the amino acid is denoted as a subscript.

Amino acid	*$RMR_{a.a./phen.}$ and conditions*[a]					
	135°—15 min			*Av.*	*RSD (%)*	*135°—4 h*
ALA_2	0.53	0.55	0.52	0.53	0.6	0.32
GLY_2	0.50	0.47	0.42	0.48	1.3	0.00
GLY_3	0.09	0.11	0.08	0.09	3.3[b]	0.46
VAL_2	0.89	0.84	0.88	0.87	0.6	0.51
LEU_2	0.94	0.93	0.95	0.94	0.1	0.88
$ILEU_2$	1.07	1.06	1.04	1.06	0.2	1.04
PRO_2	0.62	0.64	0.58	0.61	1.6	0.36
SER_3	0.98	0.94	0.95	0.96	0.3	1.00
THR_3	1.04	1.09	1.04	1.06	0.5	0.96
$HyPRO_3$	1.12	1.09	1.20	1.14	1.7	0.96
ASP_3	0.86	0.84	0.87	0.82	0.2	0.80
MET_2	0.53	0.59	0.57	0.56	1.9	0.52
$CYSH_3$	0.51	0.50	0.54	0.52	1.2	0.21
PHE_2	1.09	1.07	1.07	1.08	0.1	0.93
TYR_3	1.52	1.51	1.55	1.53	1.3	1.44

[a] Comparison of silylation in a closed tube at 135° for 15 min, and 135° for 4 h.

$$RMR_{a.a./phen.} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$$

Av. RSD (%) = 0.80.

[b] GLY_3 is not included.

TABLE VI

RELATIVE MOLAR RESPONSE OF THE TMS AMINO ACIDS

Optimum conditions, 135°—4 h. The number of TMS groups attached to the amino acid is denoted as a subscript.

Amino acid	*$RMR_{a.a./phen.}$ and conditions*[a]					
	135°—4 h			*Av.*	*RSD (%).*	*135°—15 min*
GLU_2	0.52	0.56	0.53	0.54	1.1	0.15
GLU_3	0.14	0.12	0.14	0.13	6.1[b]	0.53
ARG_3	0.04	0.00	0.00	0.01	—	0.02
ARG_4	0.54	0.50	0.48	0.51	2.4	0.03
LYS_3	0.00	0.00	0.00	0.00	—	0.20
LYS_4	0.86	0.89	0.89	0.88	2.3	0.10
HIS_3	0.55	0.54	0.55	0.55	1.8	0.31
TRY_3	0.87	0.87	0.86	0.87	1.1	0.72
TRY_2	0.16	0.17	0.15	0.16	5.0[b]	0.67
CYS_4	0.80	0.84	0.79	0.81	0.1	0.48

[a] Comparison of silylation in a closed tube at 135° for 15 min, and 135° for 4 h.

$$RMR_{a.a./phen.} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}.$$

Av. RSD (%) = 1.25.

[b] GLU_3 and TRY_2 are not included.

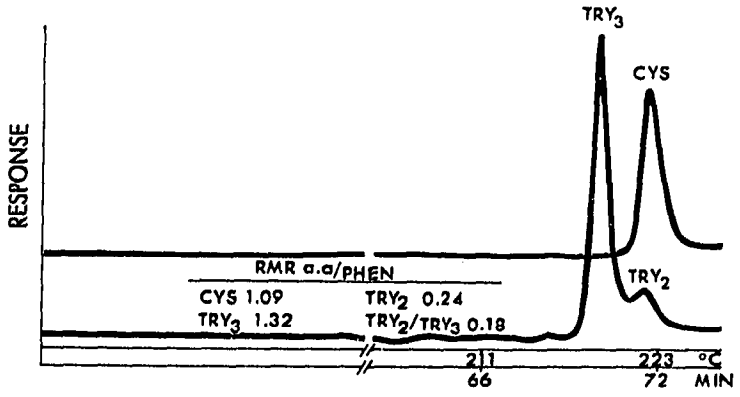


Fig. 5. GLC chromatogram of TMS tryptophan and cystine; 135° for 4 h. BSTFA/CH_3CN ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

TABLE VII

RELATIVE MOLAR RESPONSE OF TMS AMINO ACID DERIVATIVES AS A FUNCTION OF REACTION TIME

Closed tube silylation at 135°. Molar ratio of BSTFA/Amino acid = 30. BSTFA/CH_3CN ratio, 1:1.

Amino acid	*$RMR_{a.a./phen.}$ and silylation time*[a]								
	15 min			*Av.*	*2 h*	*4 h*		*Av.*	*6 h*
GLY_2	0.50	0.46	0.46	0.48	0.00	0.00	0.00	0.00	0.00
GLY_3	0.08	0.09	0.11	0.09	0.41	0.37	0.46	0.42	0.46
GLU_2	0.15	0.18	0.12	0.15	—	0.52	0.56	0.54	—
GLU_3	0.54	0.53	0.51	0.53	—	0.14	0.12	0.13	—
ARG_3	0.03	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00
ARG_4	0.01	0.28	0.15	0.14	0.26	0.54	0.50	0.52	0.48
LYS_3	0.07	0.18	0.37	0.21	0.01	0.00	0.00	0.00	0.00
LYS_4	0.10	0.13	0.07	0.10	0.65	0.86	0.84	0.85	0.80
HIS_3	0.19	0.42	0.32	0.31	0.53	0.54	0.55	0.55	0.42
TRY_3	0.88	0.80	0.47	0.72	—	0.87	0.87	0.87	—
TRY_2	0.46	0.64	0.96	0.67	—	0.16	0.19	0.18	—
CYS_4	0.36	0.51	0.51	0.57	0.86	0.78	0.84	0.82	0.78

[a] $RMR_{a.a./phen.} = \dfrac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$.

At a 6-h reaction time, some of the RMR values decreased. The best value for glycine as GLY_2 was at a reaction time of 15 min. On standing, GLY_2 changed into GLY_3, which had a retention temperature between that of isoleucine and proline. Therefore, it is essential that glycine remains as GLY_2. The silylation temperature should be held to 135° ± 2°, and the reaction time to 15 min ± 1 min. The silylated sample must be cooled within 3 min, otherwise glycine changes from GLY_2 to GLY_3. These conditions are not as critical for the other amino acids in the group of fourteen. At the silylation conditions of 135° for 4 h, the reaction time can be varied ± 10 min. The temperature should again be held to 135° ± 2°.

Stability of TMS amino acid derivatives

An important factor considered in this investigation was the stability of the

TMS amino acid derivatives on standing in a closed vial after silylation. The samples were silylated under the optimum conditions as determined earlier, and then analyzed over a seven-day period. Care was exercised to exclude moisture from entering into the reaction tube. Following silylation, and between chromatographic analyses, the silylated samples were held in the teflon-capped reaction vials at room temperature. The vials were opened only long enough to remove the sample for injection into the gas chromatograph. Stability of the TMS amino acid derivatives was evaluated by comparison of the RMR values as a function of standing time. The results are presented in Table VIII. Only glycine showed a lack of stability. It is necessary that the analysis for this amino acid be made within 3 h.

TABLE VIII

STABILITY OF THE TMS AMINO ACID DERIVATIVES AS A FUNCTION OF TIME

Sample held in closed tube at room temperature. Closed tube silylation at 135°—15 min and 135°—4 h. Each value is an average of at least two independent runs. RMR with respect to phenanthrene as internal standard.

Amino acid	*$RMR_{a.a./phen.}$ and time*					
	0	*3 h*	*8 h*	*24 h*	*3 days*	*7 days*
Silylation at 135°—15 min						
ALA_2	0.49	0.50	0.51	0.48	0.48	0.52
GLY_2	0.48	0.47	0.40	0.22	0.08	0.03
GLY_3	0.09	0.12	0.29	0.48	0.54	0.59
VAL_2	0.81	0.84	0.85	0.80	0.83	0.86
LEU_2	0.94	0.93	0.97	0.95	0.96	0.95
$ILEU_2$	1.06	1.11	1.13	0.99	1.09	1.12
PRO_2	0.56	0.58	0.54	0.60	0.58	0.56
SER_3	0.92	0.95	0.96	0.95	0.96	0.93
THR_3	0.98	1.02	0.99	1.03	0.98	0.98
$HyPRO_3$	1.07	1.09	1.04	1.08	1.12	1.02
ASP_3	0.85	0.84	0.87	0.84	0.88	0.82
MET_2	0.82	0.86	0.83	0.87	0.81	0.81
$CYSH_3$	0.52	0.53	0.55	0.55	0.54	0.56
PHE_2	1.08	1.07	1.03	1.04	1.12	1.08
TYR_3	1.46	1.52	1.50	1.48	1.53	1.46
Silylation at 135°—4 h						
GLU_2	0.54	0.53	0.59	0.59	0.55	0.53
GLU_3	0.13	0.14	0.15	0.15	0.13	0.10
ARG_4	0.55	0.53	0.60	0.56	0.61	0.58
LYS_4	0.88	0.92	0.93	0.98	1.01	1.02
HIS_3	0.52	0.56	0.56	0.54	0.51	0.47
TRY_3	0.87	0.86	0.87	0.89	0.86	0.82
TRY_2	0.16	0.15	0.13	0.17	0.13	0.08
TRY_2/ TRY_3	0.18	0.17	0.15	0.19	0.15	0.10
CYS_4	0.81	0.81	0.88	0.91	0.93	0.83

Quantitative analysis of a synthetic amino acid mixture

To establish the quantitation of the GLC analysis of the TMS amino acids, a mixture containing known amounts of selected amino acids was analyzed. The syn-

thetic mixture contained the following amino acids and amounts in 100 ml of 0.05 *N* HCl:

Amino acid	*mg*
Glutamic acid	57.6
Arginine	45.1
Lysine	41.0
Histidine	49.2
Tryptophan	50.6
Cystine	48.4

Three (2.0 ml) aliquots of the solution were placed in three culture tubes, dried, then derivatized, chromatographed, and electronically integrated as described in the section EXPERIMENTAL.

The quantity of each amino acid in the samples was calculated using the relative molar response values, $RMR_{a.a./phen.}$, obtained from the analysis of a standard amino acid solution with phenanthrene as I.S.

The per cent recovery of each amino acid was determined by comparison of the experimental results with the actual, or theoretical, values. The per cent recovery of the six selected amino acids is given in Table IX. The recoveries ranged from 93.6 to 105.9% and were considered good.

The GLC chromatograms for the recovery of the TMS amino acids in a mixture (Table IX) are shown in Figs. 6 and 7.

TABLE IX

GLC ANALYSIS OF TMS AMINO ACIDS IN A MIXTURE

Amino acid	*Milligrams of amino acid*				
	Added	*Recovered by GLC*[a,b]		*Av.*	*Recovery (%)*
$GLU_2 + GLU_3$	1.152	1.186	1.169	1.169	1.174	101.9
ARG_4	0.902	0.965	0.870	0.918	0.917	101.6
LYS_4	0.820	0.838	0.773	0.783	0.798	97.3
HIS_3	0.984	1.078	1.041	1.003	1.042	105.9
$TRY_3 + TRY_2$	1.012	0.989	0.909	0.943	0.947	93.6
CYS_4	0.968	1.075	0.992	0.968	1.011	104.4

[a] Phenanthrene as internal standard.
[b] Each value represents an independent analysis.

The per cent recovery of glutamic acid was obtained by summing the peak areas of GLU_2 and GLU_3. The amount of TRY_2 which coincided chromatographically with CYS_4 was calculated as being 18% of TRY_3, and thus TRY_3 and TRY_2 were summed to give the total area of tryptophan.

An example of the calculations used to determine the recovery of the amino acids follows. Phenanthrene, as I.S., was assigned the value of unity, and the recovery for histidine in a sample was calculated by the internal standard method:

$$g_{His} = \frac{A_{His} \times GFW_{His} \times g_{I.S.}}{A_{I.S.} \times GFW_{I.S.} \times RMR_{His/I.S.}}$$

$$g_{His} = \frac{20612 \times 155.2 \times 0.000512}{17667 \times 178.22 \times 0.52}$$

$$g_{His} = 1.003 \times 10^{-3} = 1.003 \text{ mg}$$

TMS derivatives of urea, ornithine, and citrulline

Urea, ornithine, and citrulline were trimethylsilylated at 135° for 15 min and 135° for 4 h. All of the other chromatographic and instrumental conditions were the same as for the twenty amino acids. The experimental details are given on the respective Figs. 8, 9 and 10. It was observed that urea, ornithine, and citrulline all gave more than one peak. $UREA_3$ was essentially converted to $UREA_4$ at 135° for 4 h, and

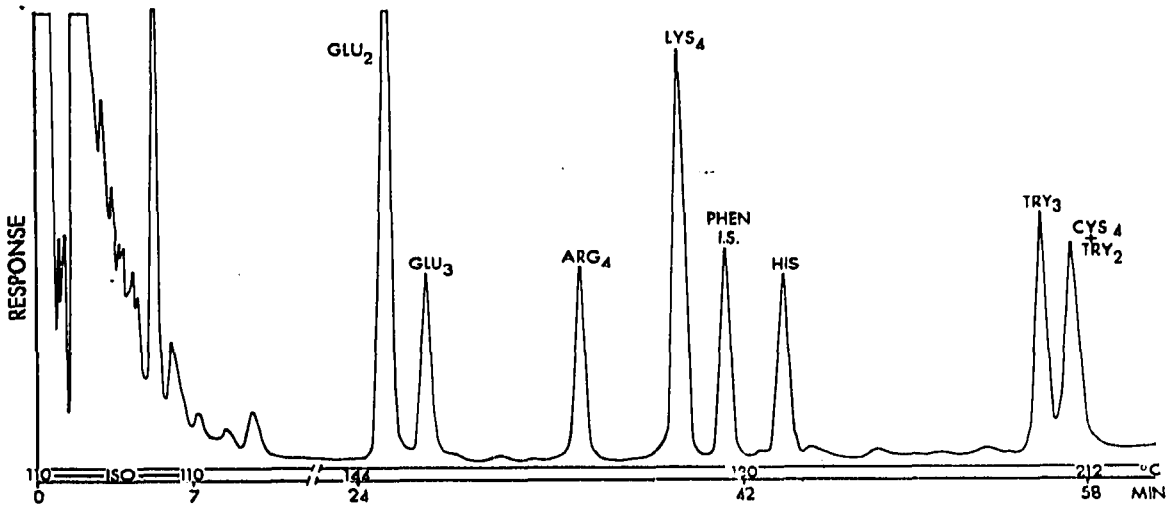


Fig. 6. GLC chromatogram of TMS amino acids; 135° for 4 h. Sample: 6 mg in 3.0 ml, 10 μg total amino acids injected (5 μl). BSTFA/CH_3CN ratio 1:1. Initial temperature 110°, 7 min hold, then 2°/min. Column, same as Fig. 2.

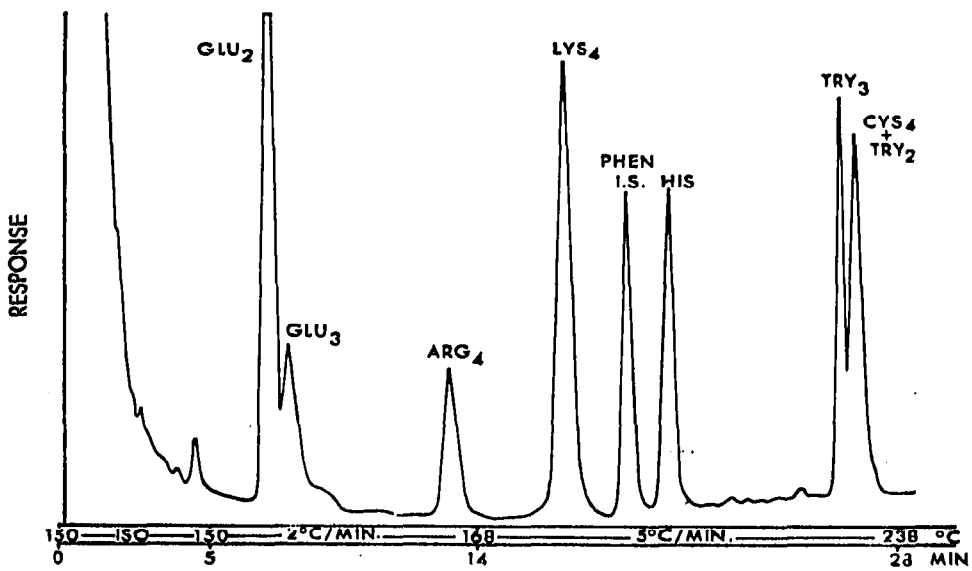
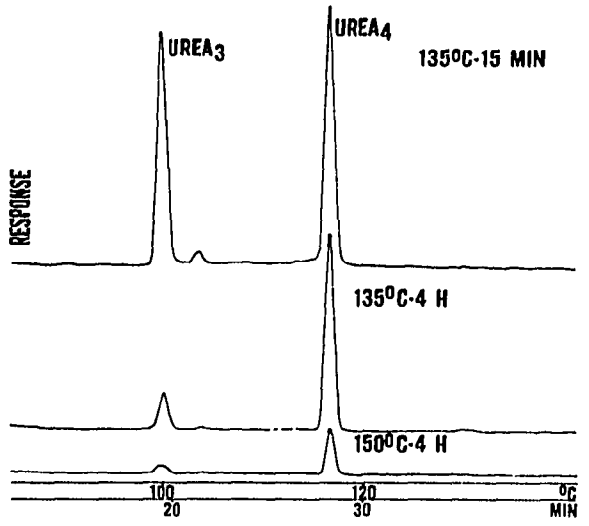


Fig. 7. GLC chromatogram of TMS amino acids; 135° for 4 h. Sample: 6 mg in 3.0 ml, 10 μg total amino acids injected (5 μl). BSTFA/CH_3CN ratio 1:1. Initial temperature 150°, 5 min hold, then 2° for 9 min, then 5°/min. Column, same as Fig. 2.



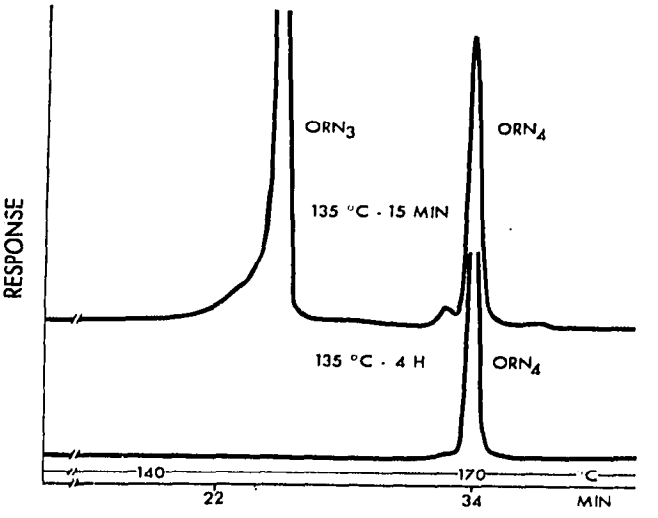


Fig. 8. GLC chromatogram of TMS urea. Sample: 1.0 μl. BSTFA/CH_3CN ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2. Attenuation: 10 × 64.

Fig. 9. GLC chromatogram of TMS ornithine. BSTFA/CH_3CN ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

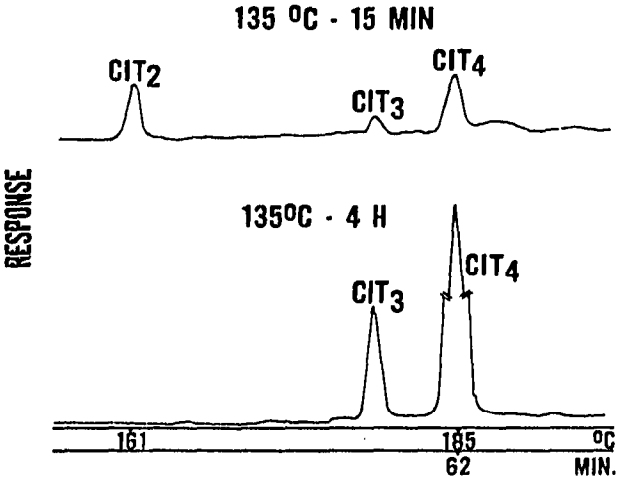


Fig. 10. GLC chromatogram of TMS citrulline. BSTFA/CH_3CN ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

at a temperature of 150° for 4 h the peaks were reduced. Trimethylsilylation of citrulline at 135° for 15 min yielded three peaks, CIT_2, CIT_3, and CIT_4, which upon heating at 135° for 4 h resulted in a loss of the CIT_2 peak and an increase in the area for the CIT_3 and CIT_4 peaks. In a similar manner, ornithine was converted from ORN_3 to ORN_4 when silylated at 135° for 4 h.

Analysis of biological materials

To establish the applicability of the entire TMS derivatization and chromatographic method for the analysis of amino acids in biological materials, ribonuclease was selected as a representative protein. Fifty milligrams of ribonuclease were hydrolyzed with 50 ml of constant boiling HCl (6 *N*) for 18 h at 105° under a nitrogen

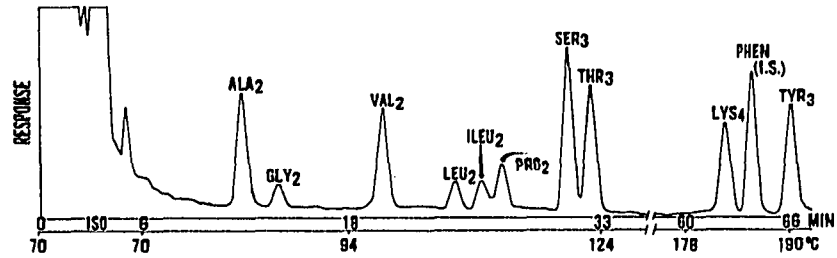


Fig. 11. GLC chromatogram of TMS amino acid derivatives of ribonuclease; 135°—15 min. Sample: 4 mg in 2.0 ml, 8 μg total amino acids injected (4 μl). Initial temperature 70°, 6 min hold, then 2°/min. Column, same as Fig. 2.

TABLE X

COMPARISON OF GAS–LIQUID AND ION-EXCHANGE CHROMATOGRAPHIC ANALYSES OF RIBONUCLEASE

Protein hydrolyzed for 18 h at 105° in a closed tube with constant boiling HCl.

Amino acid	*w/w %*				
	Gas–liquid chromatography[a]			*Av.*[b]	*Ion-exchange chromatography*[c]
Alanine	6.45	6.59	6.80	6.61	6.91
Glycine	1.56	1.71	1.62	1.63	1.78
Valine	6.43	6.60	6.28	6.44	6.33
Leucine	2.18	2.03	2.22	2.14	2.07
Isoleucine	1.63	1.71	1.65	1.66	1.84
Proline	2.72	2.63	2.71	2.69	2.96
Serine	8.91	9.20	9.13	9.08	9.01
Threonine	6.94	6.77	6.91	6.87	7.03
Aspartic acid	12.72	12.89	12.63	12.75	13.06
Methionine	3.16	3.04	2.81	3.00	3.35
Glutamic acid	11.49	11.62	11.35	11.49	11.37
Phenylalanine	3.47	3.41	3.21	3.36	3.24
Arginine	5.01	5.27	4.96	5.08	4.92
Lysine	10.39	10.49	10.21	10.36	10.25
Tyrosine	6.29	6.67	6.49	6.48	6.81
Histidine	3.49	3.42	3.21	3.40	3.69
Cystine	6.04	6.21	5.89	6.05	5.88
				99.09	100.50

[a] TMS derivatives.
[b] Average of three independent samples. Phenanthrene as internal standard.
[c] Norleucine as internal standard.

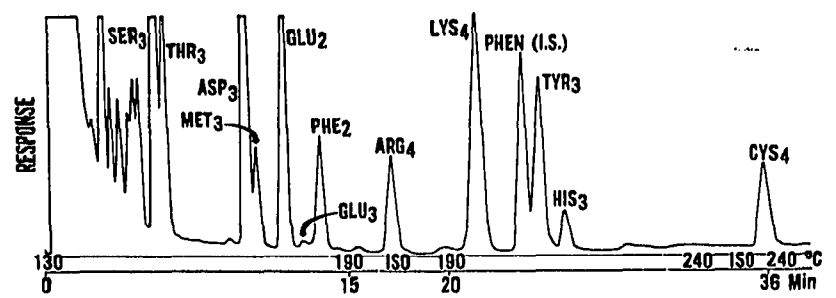


Fig. 12. GLC chromatogram of TMS amino acid derivatives of ribonuclease; 135°—4 h. Sample: 4 mg in 2.0 ml, 8 μg total amino acids injected (4 μl). Initial temperature 130°, 4°/min for 15 min, then 5 min hold, then 4°/min to 240°. Column, same as Fig. 2.

atmosphere in a closed tube. After evaporating the sample to dryness at room temperature with a rotary evaporator, the sample was transferred to a 50-ml volumetric flask and brought to volume with 0.1 *N* HCl. Five milliliter aliquots of this stock ribonuclease solution were then transferred to 16 mm × 75 mm glass reaction tubes, dried, and derivatized as described in the section EXPERIMENTAL.

The chromatograms obtained on silylation at 135° for 15 min and 4 h, respectively, are presented in Figs. 11 and 12. The data obtained from three independent GLC analyses are given in Table X, and the results are in good agreement with those from classical ion-exchange analysis.

Preliminary investigations on the GLC analysis of cation and anion-exchange cleaned human urine by the TMS technique have shown that some problems still exist. The urine samples contained a large amount of glycine, and difficulty in obtaining a single peak for glycine was noted. Both the di-trimethylsilyl (GLY_2) derivative and the tri-trimethylsilyl (GLY_3, *ca.* 10%) derivative were obtained when the samples were derivatized at 135° for both 10 and 15 min. The GLY_3 peak interfered with the resolution of TMS leucine and TMS proline due to the large quantity of glycine in the sample. Further investigations are needed to obviate this problem.

CONCLUSIONS

The experiments conclusively demonstrate that the twenty natural protein amino acids and other nonprotein amino acids can be quantitatively analyzed as their TMS derivatives by GLC. This method offers important advantages in terms of simplicity and speed, as derivatization involves only the addition of reagents with no transfers. The chromatographic separation can be accomplished on a single column. Complex biological materials as urine and blood can be analyzed by this method. Research scientists will find this GLC method of the TMS amino acids valuable in their investigations of biologically important substances which contain many functional groups that can be trimethylsilylated.

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