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TRIMETHYLSILYLATION OF AMINO ACIDS*

DERIVATIZATION AND CHROMATOGRAPHY

CHARLES W. GEHRKE** AND KENNETH LEIMER***
University of Missouri, Columbia, Mo. 65201 (U.S.A.)

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SUMMARY

The major aim of this investigation was to extend the work of GEHRKE *et al.*[3] to achieve a single derivatization, single injection method for the analysis of complex biological substances for the twenty protein amino acids as their trimethylsilyl derivatives.

The chromatographic resolution of the trimethylsilyl esters of the twenty protein amino acids can be achieved on a single column of 10 w/w% OV-11 on 100/120 mesh Supelcoport in 60–80 min (6 m × 2 mm I.D.). Phenanthrene, decanoic acid, and fluorene are suitable internal standards as they are completely separated from the twenty protein amino acids. With a 2 m × 1.6 mm I.D. column separation was achieved in 42 min.

The best reaction conditions for the quantitative silylation of the twenty amino acids were investigated. Seventeen of the amino acids can be reproducibly converted in a closed reaction tube to the trimethylsilyl derivatives in 15 min at 150°. However, for glycine, arginine, and glutamic acid, 2.5 h at 150° are necessary for reproducible derivatization. It is recommended that silylation for 2.5 h at 150° be used for all twenty protein amino acids. A significant advantage is that the trimethylsilyl derivatives can be formed in a single step derivatization with no transfers, evaporations, or reagent additions during their formation.

The N-trimethylsilyl amino acid esters were found to be stable for a period of eight days when stored at room temperature in a tightly capped vial. Glycine, cysteine, tyrosine, and lysine were the most susceptible to variations in $R.M.R._{a.a./I.S.}$.

The data for amino acids in ribonuclease, β-casein, K-casein, soybean meal, and blood are in good agreement with the values obtained by the classical ion-exchange method, and establishes the use of the trimethylsilyl/gas–liquid chromatographic method for the quantitative analysis of biological materials.

Twelve non-protein amino acids were derivatized with precision at 150° for

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** Professor. Experiment Station Chemical Laboratories.

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15 min with bis(trimethylsilyl)trifluoroacetamide in acetonitrile as solvent, with relative retention time and relative weight response values with respect to fluorene (I.S.) being reported on a 2 m × 4 mm I.D. glass column of 10 w/w% OV-7 on 100/120 mesh Supelcoport.

INTRODUCTION

BLAU[1], in a general review of amino acid analysis by gas–liquid chromatography (GLC), presents a particularly good discussion of the history of the trimethylsilyl (TMS) derivative of amino acids. In 1968, STALLING *et al.*[2] introduced a new silylating reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA, U.S. Patent No. 3,415,864). This compound is a volatile and powerful reagent for the derivatization of amino acids. GEHRKE and coworkers demonstrated that reproducible derivatives could be obtained for all twenty protein amino acids. Recently, GEHRKE *et al.*[3] published a comprehensive method for the twenty protein amino acids as the TMS derivatives using BSTFA as the silylating reagent. In this paper it was reported that problems were experienced with samples containing large amounts of glycine due to the interference of the second glycine (Gly_3) peak with the resolution of isoleucine and proline.

In early 1970, BERGSTROM *et al.*[4] reported on the trimethylsilylation of amino acids. These workers used BSTFA with and without acetonitrile as solvent at 125° for 15 min and obtained chromatographic peaks for eighteen protein amino acids. They reported on the mass spectra of the two derivatives each for glycine and lysine. The results for the mass spectra were in good agreement with the structural assignments given by GEHRKE *et al.*[3].

GEHRKE AND LEIMER[5] have shown the effect of polar and nonpolar solvents on the derivatization of amino acids using BSTFA. Also, they reported preliminary work on a chromatographic column which gave excellent resolution for the twenty protein amino acids as the TMS derivatives.

Recently, SMITH *et al.*[6] reported on the decomposition of the TMS amino acids due to the solid support of the chromatographic column. They found Gas-Chrom Q and H.P. Chromosorb W produced the least decomposition when using TMS lysine as a model compound.

In 1969, SMITH AND SHEWBART[7] reported on a quantitative comparison of trimethylsilylating reagents for protein amino acids using phenylalanine, tyrosine, and lysine as representative amino acids. This work compared trimethylsilyldimethylamine, trimethylsilyldiethylamine, bis(trimethylsilyl)trifluoroacetamide, bis(trimethylsilyl)acetamide, N-methyl-N-trimethylsilylacetamide, and trimethylsilylimidazole under various reflux conditions. This group concluded that the trimethylsilylamines were preferred to the trimethylsilylamides due to the stability of the resulting derivative solution and the greater volatility of the trimethylsilylamines. These investigators failed to obtain optimum silylation conditions for the trimethylsilylamides; as polar solvents were not used, such as acetonitrile; which greatly enhances the silylating power of these amide reagents. GEHRKE *et al.*[3] reported that the derivatized solution using bis(trimethylsilyl)trifluoroacetamide was stable for at least seven days when protected from moisture.

This study reports on the derivatization reaction conditions, chromatographic separation, and stability of the TMS derivatives of the amino acids. Comparative data are presented on GLC and classical ion-exchange analysis of proteins and biological fluids.

EXPERIMENTAL

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

Reagents and materials

Acetonitrile was purchased from Mallinckrodt Chemical Works, St. Louis, Mo., and was of "Nanograde purity". This reagent was used as received.

All amino acids were obtained from Mann Research Laboratories, New York, N.Y., and were "Mann Assayed" chromatographically pure.

Column packing for TMS derivatives are available from Analytical Biochemistry Labs., P.O. Box 1097, Columbia, Mo. 65201, and Regis Chemical Co.

Methylene chloride was purchased from Mallinckrodt Chemical Works, and was of "Analytical Reagent" grade. It was dried by refluxing over anhydrous calcium chloride and distilled into an all glass inverted top bottle to protect from atmospheric moisture.

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Regis Chemical Company, Chicago, Ill., 1101 N. Franklin St.

Fisher scientific thermally stabilized bath oil No. O-2 was used in the constant temperature bath.

SE-30, OV-7, OV-11, OV-17, and OV-22 substrates, and Supelcoport (100/120 mesh) support were purchased from Supelco, Inc., Bellefonte, Penn.

Dimethyldichlorosilane, decanoic acid, phenanthrene, and fluorene were obtained from Eastman Organic Chemicals, Rochester, N.Y., and were of highest purity available.

Ribonuclease A from bovine pancreas, crystallized 5 ×, Type I-A protease free, essentially salt free, activity of 70 Kunitiz units per mg, was obtained from Sigma Chemical Co., St. Louis, Mo.

K-casein was isolated by the method of CRAVEN AND GEHRKE[8]. *β*-Casein was isolated by the method of GEHRKE *et al.*[9].

Stock solutions

The standard amino acid solution used contained 0.100 mg/ml of each amino acid in 0.05 *N* HCl.

Internal standard stock solutions of phenanthrene, fluorene, and decanoic acid were maintained at 0.100, 0.200, 0.500, and 1.000 mg/ml in acetonitrile.

Apparatus and glassware

The oil bath, in which the closed tube trimethylsilylation reaction was conducted, consisted of a $3\frac{1}{2} \times 4 \times 6$ in. aluminium pan supported on a magnetic stirrer to maintain uniform temperature of the oil bath. Temperature control was achieved with 2 (100 W) heaters and a variac.

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 tube.

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

A Mark I magnetic stirring unit with controlled hot plate was purchased from Cole-Parmer, Chicago, Ill., and was used as a dry heat bath for evaporations under a stream of filtered nitrogen gas.

Instrument

A MicroTek Model MT-220 automatic sequential programmed-temperature instrument equipped with a four column oven, four flame ionization detectors and two differential electrometers was used for this investigation. A Varian Model 30 recorder was used for the chart presentation. The chromatograph was equipped with a Hewlett-Packard 3370A digital integrator and an Infotronics CRS-104 digital integrator for determination of peak areas for quantitative work.

Column preparation

An accurately weighed amount of Supelcoport (100/120 mesh) was placed in a ribbed round bottom flask and just covered with methylene chloride. Then, an appropriate amount of the desired loading of substrate was dissolved in methylene chloride and added to the flask, (*i.e.*, for a 10 w/w% OV-11 packing, weigh 20.00 g Supelcoport and 2.22 g OV-11). The solvent was then slowly evaporated (1 to 2 h) at room temperature, using a rotary evaporator until just damp and then immersed in a 60° water bath under full vacuum until no odor of methylene chloride remained. The dry packing was added to a glass column which had been silylated by the procedure recommended by Applied Science Laboratories, Inc.[10]. The glass column was filled with a solution of 10 v/v% dimethyldichlorosilane in toluene and allowed to stand at least 15 min. The solution was removed and the column filled with methanol, followed by two washes with acetone and drying of the column. While adding the packing to the column, gentle tapping was used to obtain a uniform distribution. For long columns, it was necessary to use a vacuum to pack the column. This was accomplished by placing a glass wool plug in one end and pulling a vacuum on that end and adding the packing in small portions through the open end and gently tapping. A plug of silanized spun glass wool was then placed in the open end of the column to hold the column packing in place. The column was placed in a column oven and flushed for 30 min with carrier gas and then no flow conditioned at 325–330° for 12 to 15 h. The oven was then cooled to room temperature and a flow of 10–15 ml/min was used for the remainder of the conditioning. The oven was programmed to 300° at 1°/min and allowed to remain undisturbed at this temperature for at least 24 h.

Trimethylsilylation derivatization method

An aqueous aliquot containing from 0.5 mg to 6 mg of total amino acids was added to a silylation reaction tube. The solution was evaporated just to dryness, by placing the tube in a 70° dry, or sand bath and passing a regulated stream of filtered nitrogen into the tube, then 0.5 ml of methylene chloride was added and evaporated

just to dryness to ensure the complete azeotropic removal of water. This step was repeated at least once. An accurately known amount of one of the internal standards in acetonitrile (phenanthrene, decanoic acid, or fluorene) corresponding to about the amount of each amino acid, was pipetted into the tube such that there was 0.25 ml of acetonitrile for each mg of total amino acids. Then, 0.25 ml (30 molar excess) of BSTFA was added for each 1 mg of total amino acids and the tube was closed securely with a teflon lined cap and placed in an ultrasonic bath for 1 min. Trimethylsilylation of the amino acids was done by heating the tube for 2.5 h at 150° in an oil bath.

Comments on the GLC method

1. A properly derivatized equal weight standard should have a ratio of approximately 15:1 for Gly_3:Gly_2; 10:1 for Glu_2:Glu_3; no Lys_3 peak, but a large Lys_4 peak; and an Arg_4 peak approximately one-half the size of the lysine peak. If the ratios are not correct, the sample should either be heated at a higher temperature or for a longer time.

2. While the amounts of reagents given above work quite satisfactorily, it is sometimes desirable to double the amounts of acetonitrile and BSTFA compared to the total weight of amino acids. This is probably necessary in some cases due to the quality of the BSTFA reagent or inadequate drying of the sample.

3. Poor results are often obtained if the sample is not properly dried. This is easily accomplished by placing the sample tube in a 60 to 70° sand bath and passing a stream of pure dry nitrogen into the tube. The sample should not be heated in the presence of oxygen, as color develops in the sample and poor results are obtained. When the sample appears to be just dry, 0.5 ml of methylene chloride is added and taken just to dryness. This step can be repeated several times as needed to azeotropically remove the last traces of water. A properly dried standard should be either white or colorless as a film on the walls of the tube.

4. The heating of the tube during derivatization should be done at 150 ± 5° in an oil bath. If a sand bath or other heating device is used, the optimum time and temperature for derivatization will have to be determined; as the rate of heat transfer from solid to solid, or gas to solid, are greatly different from those of a liquid to solid. The use of a sand bath has been reported to give difficulty in the derivatization. The derivatization tube should be only 1/4 full and should not be immersed in an oil bath above the level of liquid in the tube. This allows for reflux of the solvent without build-up of excessive pressure. An oil bath is recommended.

5. Fluorene, phenanthrene, and decanoic acid have been used, interchangeably, as internal standards in this investigation. The authors prefer decanoic acid as an internal standard, as occasionally, fluorene and phenanthrene are not separated from arginine and histidine, respectively. The separation of fluorene and phenanthrene are very dependent on the chromatographic column, temperature, and program rate used, hence, the authors' preference for decanoic acid. No separation difficulties have been experienced with decanoic acid. Its use is shown in Figs. 8 and 9.

6. Performance blanks of all reagents should be made to check the purity of all reagents. It is especially important to check the purity of the BSTFA as some commercial lots have extraneous peaks which may interfere with the analysis.

7. Iron must be carefully excluded from the sample to obtain accurate and reproducible values for methionine.

8. Direct on-column injection or the use of glass-lined injection ports eliminates decomposition of threonine and serine, which was observed when all-metal flash heaters were used.

Chromatographic column

9. For complete resolution of all twenty amino acids, a 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm I.D., glass column is recommended. However, for separation only of methionine, glutamic acid, phenylalanine, arginine, lysine, tyrosine, histidine, tryptophan, and cystine, a 10 w/w% OV-7 substrate on 100/120 mesh Supelcoport, 2 m × 2 mm I.D., glass column can be used. Note the separation achieved with a 2 m × 1.6 mm, 10% w/w OV-11, column in 42 min (Fig. 10).

RESULTS AND DISCUSSION

Chromatographic separation studies

The chromatographic separation presented by GEHRKE *et al.*[3] was hard to reproduce due to the great dependence of the separation of histidine, phenanthrene, and tyrosine on the substrate loading. At substrate loadings slightly higher than they recommended, phenanthrene and tyrosine are poorly resolved, while at slightly lower loadings histidine and phenanthrene were not separated. It was noted that the 2:1 ratio of OV-7/OV-22 used by GEHRKE *et al.*[3] has the same methyl/phenyl compo-

TABLE I

EFFECT OF SUBSTRATE ON SEPARATION OF TMS AMINO ACIDS

All loadings were 10 w/w% on 100/120 mesh Supelcoport. All columns were glass, 4 m × 2 mm I.D.

Amino acid	*Relative retention time and substrate*				
	SE-30	*OV-7*	*OV-11*	*OV-7/ OV-22 (2:1)*	*OV-17*
Alanine	0.351	0.346	0.358	0.360	0.331
Valine	0.477	0.470	0.478	0.488	0.450
Leucine	0.530	0.523	0.529	0.543	0.500
Isoleucine	0.549	0.541	0.547	0.562	0.518
Glycine (Gly_3)	0.567	0.560	0.564	0.581	0.557
Proline	0.577	0.569	0.582	0.586	0.579
Serine	0.600	0.610	0.596	0.633	0.583
Threonine	0.607	0.599	0.611	0.621	0.572
Hydroxyproline	0.703	0.706	0.693	0.710	0.645
Aspartic acid	0.716	0.706	0.707	0.729	0.675
Methionine	0.735	0.725	0.724	0.743	0.693
Cysteine	0.744	0.734	0.733	0.762	0.702
Glutamic acid	0.767	0.757	0.756	0.786	0.724
Phenylalanine	0.800	0.789	0.787	0.819	0.754
Arginine	0.860	0.858	0.844	0.876	0.816
Lysine	0.893	0.912	0.890	0.933	0.860
Tyrosine	0.953	0.940	0.933	0.976	0.899
Histidine	0.977	0.963	0.956	0.995	0.921
Tryptophan	1.123	1.138	1.124	1.200	1.096
Cystine	1.172	1.156	1.142	1.205	1.101
Phenanthrene	1.000	1.000	1.000	1.000	1.000

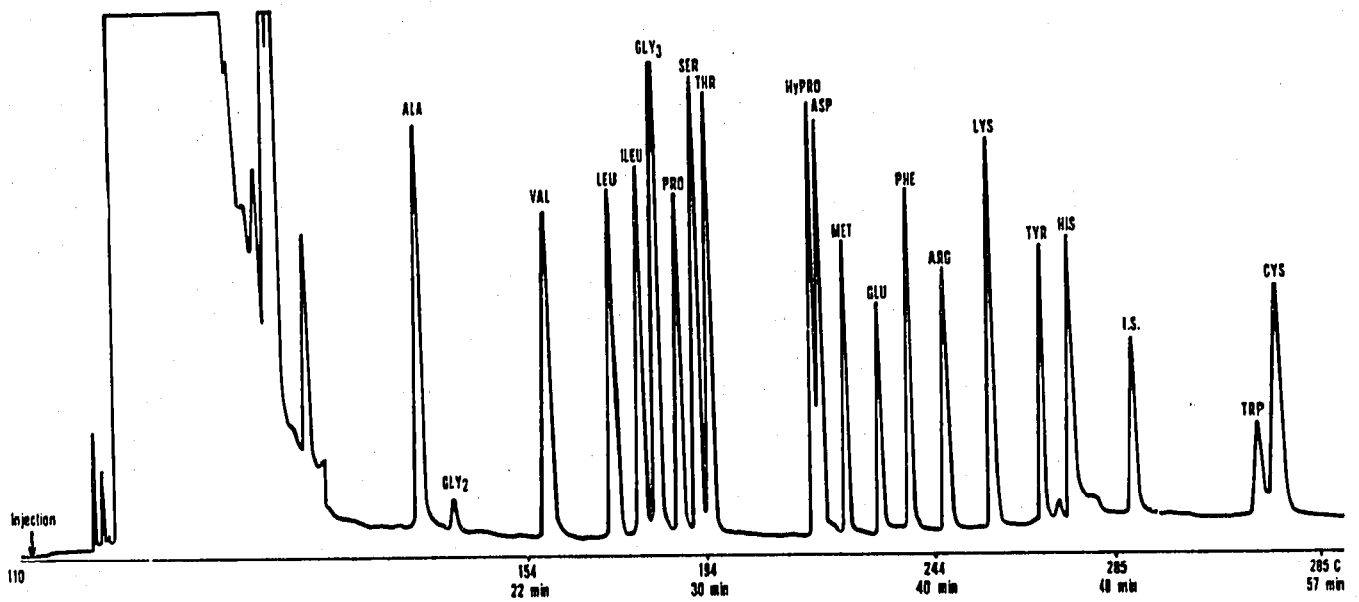

Fig. 1. Chromatogram of TMS protein amino acids. Sample: 2.0 mg in 1.0 ml. 1.0 μg each amino acid injected. BSTFA–CH_3CN (1:1), 150° for 2.5 h. Column: 10% OV-11 on Supelcoport 100/120 mesh. 6 m × 2 mm I.D. Conditions: injector, 275°; detector, 300°, initial temperature 110°, 2°/min for 22 min, 5°/min to 285°; carrier gas N_2, 20 ml/min. I.S. = phenanthrene.

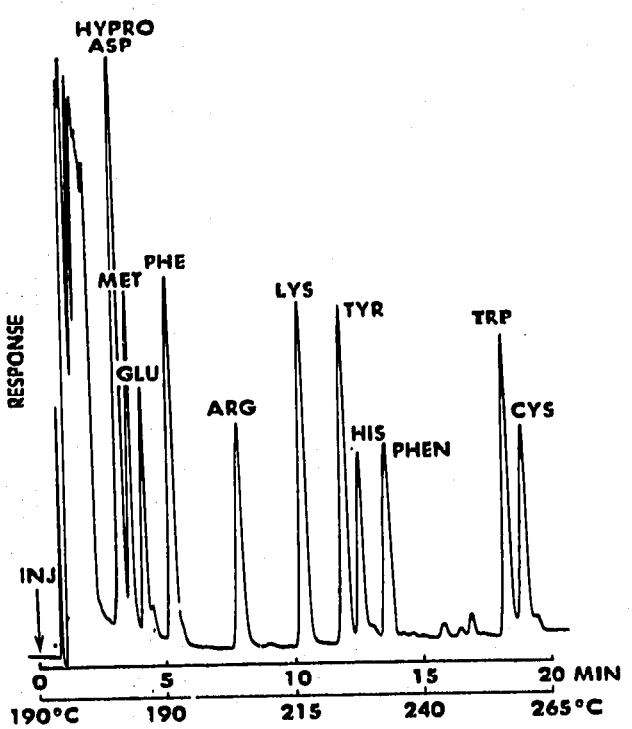

Fig. 2. Gas–liquid chromatogram of selected amino acids. Sample: 0.2 mg each of 19 amino acids in 2.0 ml BSTFA–CH_3CN (1:1). Closed tube silylation; 150° for 2.5 h; 5 μl injected. Column: 10 w/w% OV-7 on 100/120 mesh Supelcoport, 2 m × 2 mm I.D. glass. Initial temperature 190° for 5 min, then 5°/min, each peak *ca.* 500 ng.

sition as OV-11 which is a 35% phenyl and 65% methyl siloxane. Chromatography on OV-11 (10 w/w%) instead of on the mixed substrate phase resulted in a superior resolution of isoleucine, glycine (Gly_3), and proline, but did not show sufficient improvement to overcome the difficulty with samples containing large amounts of glycine.

The effect of column support, length, substrate, and loading were then investigated to find a column capable of resolving all twenty of the protein amino acids. The solid supports used were H.P. Chromosorb G, H.P. Chromosorb W, and Supelcoport. Supelcoport was found to be the most inactive solid support with respect to reproducible elution of the TMS amino acid derivatives using OV-11 at 6% loading. Therefore, Supelcoport was selected as the solid support in further studies. The effect of different substrates at 10 w/w% is presented in Table I. This table shows that OV-11 gave the best overall resolution, while for some particular separations other substrates were preferred. SE-30 would be preferred for the separation of tryptophan and cystine. The best separation for cysteine and methionine was achieved with the mixed liquid phase (OV-7/OV-22, 2:1), whereas OV-17 gave the best separation for hydroxyproline and aspartic acid. The best resolution for isoleucine, glycine (Gly_3), and proline was obtained on OV-11 and OV-17, while the mixed phase yielded the poorest resolution.

From the above information, it was decided that an OV-11 column, 6 m × 2 mm I.D., would be the desired column. A study was then made to determine the best loading. Loadings of 5, 7.5, 10, 12.5, and 15 w/w% were investigated. The 10 w/w% loading gave the most consistently reproducible separation and relative weight response (*R.W.R.*) values. For these reasons, a 6 m × 2 mm I.D. 10 w/w% OV-11 on 100/120 mesh Supelcoport was selected as the column of choice. The separations on such a column are illustrated in Fig. 1.

It should be noted that some difficulty was experienced in obtaining a column of this length on which histidine was eluted in a quantitative manner. For this reason it may be desirable to use a shorter column for the determination of histidine. Such a column is illustrated in Fig. 2. The short column that may be used for histidine is 2 m × 4 mm I.D. 10% OV-7 on 100/120 mesh Supelcoport. This column may also be used to separate methionine, glutamic acid, arginine, phenylalanine, lysine, tyrosine, histidine, tryptophan, and cystine. With the long OV-11 column, on more

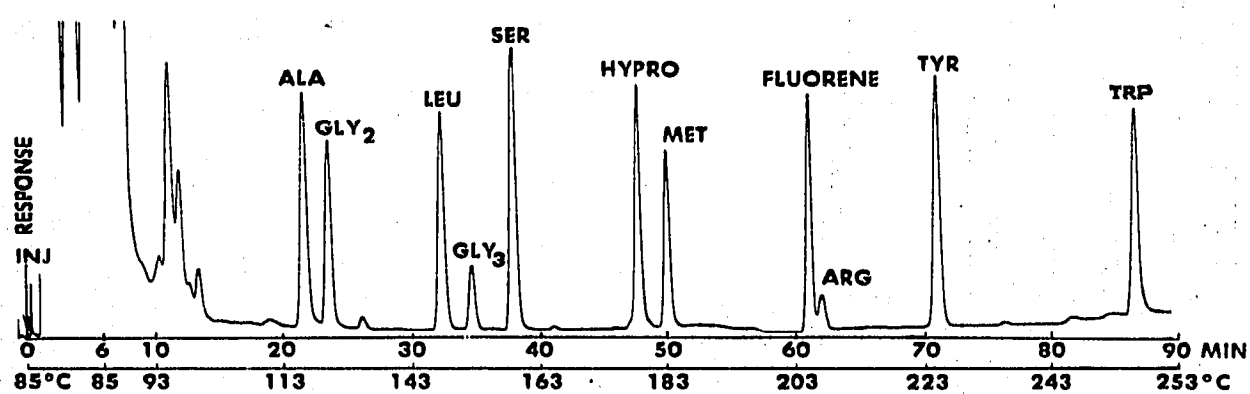

Fig. 3. Gas–liquid chromatogram of selected TMS amino acid derivatives. Sample: 0.2 mg of each amino acid in 2.0 ml CH_3CN–BSTFA (1:1). Closed tube silylation, 135° for 30 min. 6 µl injected (*ca.* 600 ng each). Column: mixed liquid phase 6.0 w/w% OV-7 and 3.0 w/w% OV-22 on 100/120 mesh Supelcoport, 2 m × 4 mm I.D. glass. Initial temperature 85°, then 2°/min.

than one occasion the authors have observed a reversal in the order of elution of hydroxyproline–aspartic acid, tryptophan–cystine, and histidine and the I.S. phenanthrene. Before the use of such a column, the order of elution of the amino acids should be checked as the factors controlling this reversal are not known.

GEHRKE *et al.*[3] reported that the injection port temperature must be at 250 to 300° or there is incomplete elution of the less volatile derivatives. The present authors have found that with injection port temperatures as low as 180°, one still obtains good reproducible results. The lower injection port temperatures eliminate some of the solvent tailing problem observed by the previous authors. It is suggested that an injection port temperature of 230 to 250° should be maintained, but it is not necessary.

In addition, these authors[3] reported that tryptophan gave two peaks under all chromatographic conditions used. We have found that the second peak for tryptophan and for histidine is a function of the column packing. Certain columns would not give a second peak for either of these compounds, while other columns gave a second peak under all conditions. This appears to be a function of how uniform the substrate coating is on the solid support. Fig. 3 is a representative chromatogram showing only one peak for tryptophan. The columns used throughout this investigation gave only one peak for tryptophan.

Derivatization reaction conditions

GEHRKE *et al.*[3] reported that due to their conditions for chromatographic separation, two derivatizations were necessary to achieve quantitation and separation of the twenty amino acids. With the improved resolution reported here, it was reasonable that only one derivatization and one injection should be necessary to quantitate the twenty protein amino acids. To determine the optimum derivatization conditions, a time and temperature study was made for each amino acid. Times of 15 min to 4 h and temperatures of 135, 150 and 175° were investigated. Fig. 4 presents the results for Val_2, Gly_2 and Gly_3, Glu_2, Lys_4 and Arg_4 at 150° for different times. Valine is representative of sixteen of the protein amino acids in that maximum response was achieved on silylation at 150° for 15 min. For glycine, glutamic acid, lysine and arginine; silylation conditions of 2.5 h at 150° were chosen. From this study, it was concluded that 150° for 2.5 h is the optimum temperature and time for complete derivatization of a mixture of the twenty protein amino acids.

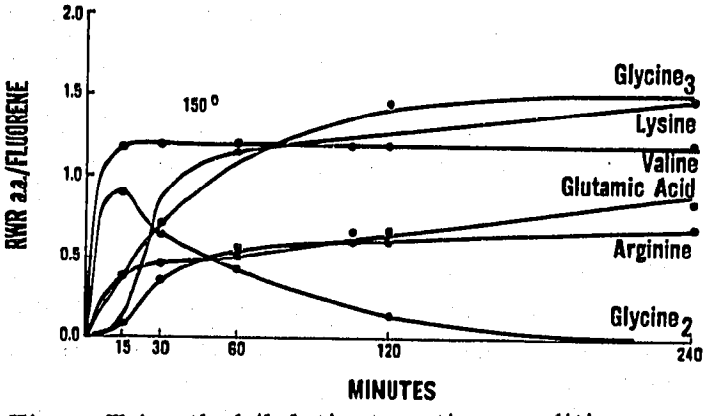

Fig. 4. Trimethylsilylation reaction conditions.

TABLE II

COMPARISON OF BSTFA AND 1% TRIMETHYLCHLOROSILANE IN BSTFA AS SILYLATING REAGENTS

Amino acid	*R.W.R.a.a./fluorene*[a,b]			
	BSTFA		*1% TMCS–BSTFA*	
	15 min	*2.5 h*	*15 min*	*2.5 h*
Alanine	1.73	1.76	1.77	1.72
Valine	1.62	1.59	1.60	1.62
Leucine	1.41	1.44	1.44	1.47
Isoleucine	1.44	1.45	1.42	1.44
Glycine	0.23[c]	1.91	0.61[c]	1.88
Proline	1.18	1.22	1.21	1.23
Serine	1.64	1.67	1.60	1.61
Threonine	1.69	1.65	1.64	1.66
Aspartic acid	1.19	1.23	1.24	1.21
Hydroxyproline	1.26	1.22	1.22	1.23
Methionine	0.76	0.77	0.74	0.76
Glutamic acid	0.07[c]	0.62	0.31[c]	0.76
Phenylalanine	1.06	1.04	1.10	1.07
Arginine	0.12[c]	0.81	0.24[c]	0.89
Lysine	1.23	1.24	1.25	1.27
Tyrosine	1.27	1.29	1.24	1.26
Histidine	0.77	0.79	0.79	0.74
Tryptophan	0.81	0.83	0.82	0.85
Cystine	0.62	0.64	0.65	0.66

[a] Each value an average of two independent samples.

[b] $R.W.R._{\text{a.a./fluorene}} = \dfrac{A_{\text{a.a.}}/\text{weight}_{\text{a.a.}}}{A_{\text{I.S.}}/\text{weight}_{\text{I.S.}}}$.

[c] Significant change.

The derivatization conditions recommended are 0.5 ml of BSTFA–acetonitrile (1:1) for each 1 mg of total amino acids and heating for 2.5 h at 150°. The molar excess of BSTFA is 30, which is that recommended by GEHRKE *et al.*[3].

CHAMBAZ AND HORNING[11,12] and CHAMBAZ *et al.*[13] have recently reported that the use of 1% trimethylchlorosilane has a catalytic effect on silylation of steroids using bis(trimethylsilyl)trifluoroacetamide. Regis Chemical Company[14] has suggested the use of 1% trimethylchlorosilane in BSTFA as a silylating reagent for active hydrogen compounds. Table II gives a comparison of BSTFA, with BSTFA containing 1% trimethylchlorosilane as a silylating reagent. The data show no large differences between the two reagents at 2.5 h at 150°, but significant differences for glycine, glutamic acid, and arginine were noted for 15 min at 150°. Thus, it was concluded that with acetonitrile as solvent, the use of 1% trimethylchlorosilane in BSTFA offers no advantage over the use of BSTFA alone for the derivatization of the twenty protein amino acids.

Relative weight response and stability of TMS amino acids

Table III gives data on the precision in the relative weight response of the amino acids with respect to phenanthrene. The average percent relative standard deviation for the twenty protein amino acids was 2.69%.

Table IV presents data to demonstrate the stability of the TMS amino acid

TABLE III

RELATIVE WEIGHT RESPONSE OF THE TMS AMINO ACIDS

Amino acid[a]	*R.W.R.*$_{a.a./phenanthrene}$[b]				
				Av.	*R.S.D. (%)*[c]
Alanine	1.75	1.77	1.67	1.73	3.04
Valine	1.68	1.68	1.64	1.67	1.53
Leucine	1.38	1.38	1.41	1.39	1.50
Isoleucine	1.44	1.47	1.48	1.46	1.40
Glycine	1.87	1.85	1.81	1.84	1.81
Proline	1.24	1.18	1.19	1.20	2.71
Serine	1.60	1.63	1.65	1.63	1.72
Threonine	1.59	1.66	1.64	1.63	2.25
Hydroxyproline	1.19	1.23	1.28	1.23	3.40
Aspartic acid	1.15	1.14	1.15	1.15	0.64
Methionine	0.86	0.85	0.77	0.83	6.14
Cysteine	0.81	0.82	0.83	0.82	1.17
Glutamic acid	0.76	0.77	0.79	0.77	1.81
Phenylalanine	1.08	1.13	1.17	1.12	3.79
Arginine	0.97	0.97	0.98	0.98	0.52
Lysine	1.37	1.43	1.41	1.40	2.01
Tyrosine	0.99	0.98	0.91	0.96	4.53
Histidine	0.80	0.76	0.86	0.81	6.16
Tryptophan	0.81	0.84	0.83	0.83	1.78
Cystine	0.65	0.57	0.63	0.62	5.82

[a] Closed tube silylation at 150° for 2.5 h.

[b] $R.W.R._{a.a./phenanthrene} = \dfrac{A_{a.a.}/\text{weight}_{a.a.}}{A_{I.S.}/\text{weight}_{I.S.}}$.

[c] Average R.S.D. (%) = 2.69.

TABLE IV

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

Amino acid[a,b]	*R.W.R.*$_{a.a./fluorene}$ *and time*[c]					
	0	*6 h*	*12 h*	*1 day*	*4 days*	*8 days*
Alanine	1.75	1.77	1.74	1.78	1.71	1.69
Valine	1.68	1.67	1.71	1.72	1.66	1.66
Leucine	1.38	1.41	1.39	1.41	1.37	1.36
Isoleucine	1.46	1.44	1.48	1.46	1.45	1.41
Glycine (Gly_3)	1.84	1.92	2.01	2.11	2.07	2.05
Proline	1.21	1.24	1.21	1.18	1.21	1.19
Serine	1.62	1.65	1.63	1.62	1.59	1.54
Threonine	1.62	1.61	1.58	1.60	1.57	1.56
Aspartic acid	1.15	1.19	1.18	1.19	1.15	1.14
Hydroxyproline	1.22	1.22	1.26	1.27	1.20	1.21
Methionine	0.86	0.86	0.88	0.84	0.87	0.85
Cysteine	0.77	0.76	0.78	0.74	0.71	0.66
Glutamic acid	0.77	0.77	0.79	0.77	0.82	0.81
Phenylalanine	1.09	1.10	1.10	1.12	1.14	1.12
Arginine	0.83	0.81	0.84	0.81	0.82	0.85
Lysine	1.26	1.30	1.32	1.36	1.31	1.34
Tyrosine	1.32	1.24	1.21	1.22	1.21	1.18
Histidine	0.81	0.82	0.84	0.80	0.81	0.78
Tryptophan	0.84	0.86	0.89	0.92	0.87	0.85
Cystine	0.66	0.63	0.62	0.64	0.65	0.61

[a] Closed tube silylation at 150° for 2.5 h. *R.W.R.* with respect to fluorene as internal standard.

[b] Sample held at room temperature.

[c] $R.W.R._{a.a./fluorene} = \dfrac{A_{a.a.}/\text{weight}_{a.a.}}{A_{I.S.}/\text{weight}_{I.S.}}$.

TABLE V

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

Protein hydrolyzed for 24 h at 110° in a closed tube with constant boiling HCl. All values are given in w/w%.

Amino acid	*Gas–liquid chromatography*[a]			*Av.*[b]	*Ion-exchange chromatography*[c]
Alanine	6.61	7.12	6.72	6.81	6.91
Valine	6.44	6.43	6.51	6.46	6.33
Leucine	2.14	1.91	1.97	2.01	2.07
Isoleucine	1.65	1.49	1.56	1.57	1.84
Glycine (Gly_3)	1.62	1.65	1.56	1.61	1.78
Proline	2.69	3.01	2.71	2.80	2.96
Serine	9.08	10.04	9.46	9.53	9.01
Threonine	6.97	7.45	7.12	7.18	7.03
Aspartic acid	12.65	13.29	12.44	12.79	13.06
Methionine	3.00	3.39	2.96	3.12	3.35
Glutamic acid	11.49	12.08	11.28	11.62	11.37
Phenylalanine	3.36	3.05	3.17	3.19	3.24
Arginine	4.98	4.67	4.72	4.79	4.92
Lysine	10.36	10.15	10.02	10.18	10.25
Tyrosine	6.48	6.56	6.53	6.52	6.81
Histidine	3.00	3.23	3.31	3.16	3.69
Cystine	6.05	6.40	5.97	6.16	5.88
Totals				99.09	100.50

[a] TMS derivatives.
[b] Average of three independent samples. Phenanthrene as internal standard.
[c] Values represent multiple analyses.

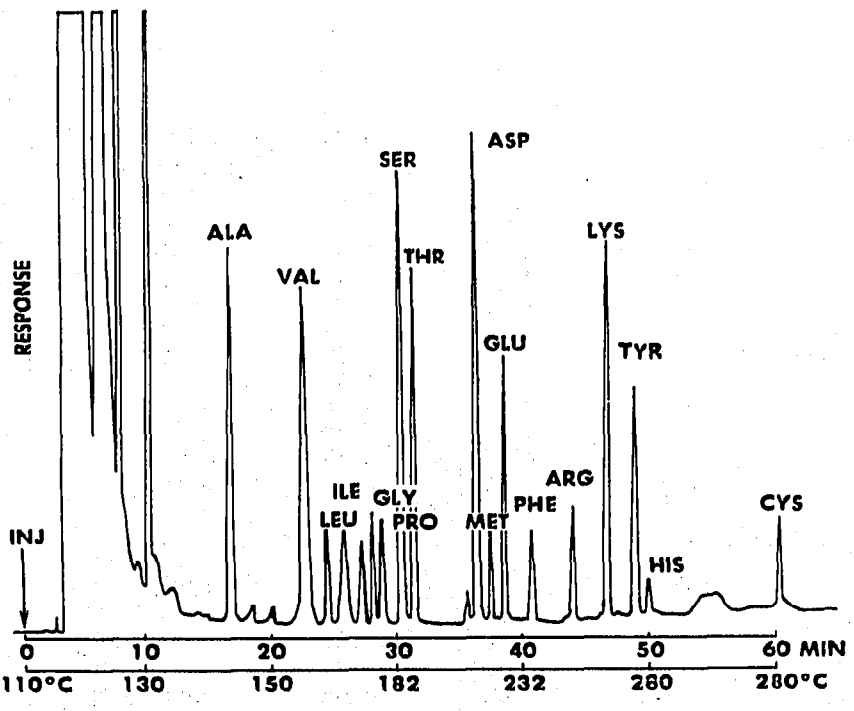

Fig. 5. Gas–liquid chromatogram of TMS amino acid derivatives of ribonuclease. Sample: 1 mg in 0.5 ml BSTFA–CH_3CN (1:1). Closed tube silylation, 150° for 2.5 h, 6 μl injected (*ca.* 15 μg total). Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm I.D. glass. Initial temperature 110°, 2°/min for 26 min, then 5°/min to 280°.

TABLE VI

COMPARISON OF GAS–LIQUID AND ION-EXCHANGE CHROMATOGRAPHIC ANALYSIS OF β-CASEIN

Protein hydrolyzed for 24 h at 110° in a closed tube with constant boiling HCl. All values are given in w/w%.

Amino acid	*Gas–liquid chromatography*[a]		*Av.*[b]	*Ion-exchange chromatography*[c]
Alanine	1.36	1.42	1.39	1.47
Valine	6.07	6.21	6.14	6.63
Leucine	9.60	9.88	9.74	10.1
Isoleucine	3.45	3.40	3.43	3.65
Glycine (Gly_3)	1.19	1.08	1.14	1.19
Proline	15.3	15.2	15.3	15.8
Serine	5.16	5.30	5.23	5.23
Threonine	3.23	3.36	3.30	3.35
Aspartic acid	4.41	4.26	4.34	4.41
Methionine	2.59	2.27	2.43	2.59
Glutamic acid	20.0	20.3	20.2	20.5
Phenylalanine	4.88	4.76	4.82	5.05
Arginine	2.38	2.09	2.24	2.28
Lysine	5.34	5.21	5.28	5.54
Tyrosine	2.41	2.58	2.50	2.63
Histidine	2.21	1.99	2.10	2.59
Cystine	—	—	—	—
Totals			89.48	93.21

[a] TMS derivatives.
[b] Average of two independent samples. Phenanthrene as internal standard.
[c] Values represent multiple analyses.

TABLE VII

COMPARISON OF GAS–LIQUID AND ION-EXCHANGE CHROMATOGRAPHIC ANALYSIS OF K-CASEIN

Protein hydrolyzed for 24 h at 110° in a closed tube with constant boiling HCl. All values are given in w/w%.

Amino acid	*Gas–liquid chromatography*[a]		*Av.*[b]	*Ion-exchange chromatography*[c]
Alanine	3.43	3.37	3.40	3.79
Valine	4.17	4.08	4.13	4.25
Leucine	4.44	4.21	4.33	4.39
Isoleucine	4.07	4.16	4.12	4.22
Glycine	1.01	1.03	1.02	1.14
Proline	7.52	7.66	7.59	7.39
Serine	4.23	4.41	4.32	4.85
Threonine	4.37	4.61	4.49	4.86
Aspartic acid	5.84	6.31	6.08	5.95
Methionine	2.28	1.71	1.99	3.51
Glutamic acid	15.42	14.96	15.17	15.81
Phenylalanine	2.81	2.94	2.88	2.91
Arginine	2.76	2.81	2.77	2.99
Lysine	5.01	5.17	5.09	5.53
Tyrosine	4.82	4.73	4.78	4.97
Histidine	1.44	1.59	1.52	1.76
Cystine	0.41	0.46	0.44	0.38
Totals			74.12	78.74

[a] TMS derivatives.
[b] Average of two independent samples. Phenanthrene as internal standard.
[c] Values represent multiple analyses.

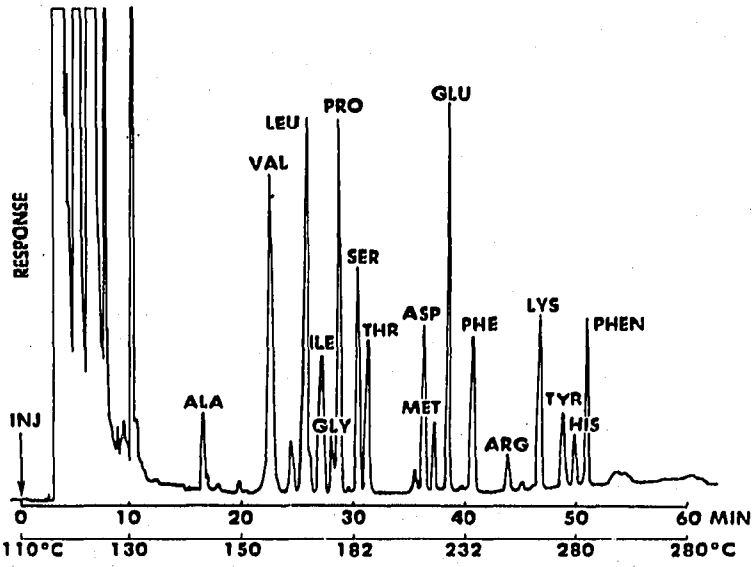

Fig. 6. Gas–liquid chromatogram of TMS amino acid derivatives of β-casein. Sample: 5 mg in 2.25 ml BSTFA/CH_3CN (1:1). Closed tube silylation, 150° for 2.5 h, 6 μl injected (*ca.* 15 μg total). Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm I.D. glass. Initial temperature 110°, 2°/min for 26 min, then 5°/min to 280°.

derivatives with respect to time in a closed tube at room temperature. In general the stability was quite good over an eight day period. The derivatives are easily maintained for 48 h without any significant change in $R.W.R._{a.a./I.S.}$. With careful handling, the derivatives have been shown to be fairly stable for eight days.

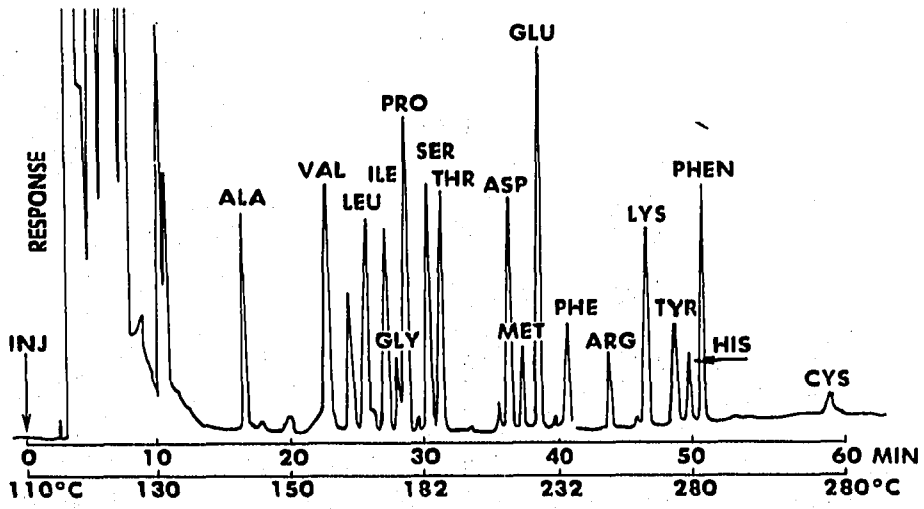

Fig. 7. Gas–liquid chromatogram of TMS amino acid derivatives of *K*-casein. Sample: 4.0 mg in 2.0 ml BSTFA–CH_3CN (1:1). Closed tube silylation, 150° for 2.5 h, 4 μl injected (*ca.* 8 μg total). Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm. I.D. glass. Initial temperature 110°, 2°/min for 26 min, then 5°/min to 280°.

Analysis of biological samples

Table V presents a comparison of the GLC analysis of a ribonuclease which was hydrolyzed for 24 h at 110°, with that obtained by classical ion-exchange chromatography. The results by these two methods of analysis were found to be in close agreement. Fig. 5 shows a chromatogram from a ribonuclease hydrolysate as the TMS derivatives.

Tables VI and VII show comparisons of the GLC results from β-casein and K-casein which were hydrolyzed for 24 h at 110°, with classical ion-exchange amino acid analysis. The data are seen to be in good agreement. Figs. 6 and 7 present the corresponding gas–liquid chromatograms of the TMS amino acid derivatives of β-casein and K-casein hydrolysates.

A comparison of classical ion-exchange data with GLC of the TMS amino acid derivatives and N-trifluoroacetyl butyl amino acid ester derivatives of a cleaned blood plasma sample are presented in Table VIII. The data by the three methods are in good agreement. Fig. 8 presents the gas–liquid chromatogram of the TMS amino acids of a cleaned blood plasma sample. The blood plasma sample was cleaned according to the procedure of ZUMWALT *et al.*[15].

A comparison of classical ion-exchange data with GLC of the TMS and N-

TABLE VIII

AMINO ACID ANALYSIS OF BOVINE BLOOD PLASMA

Blood plasma cleaned by cation exchange.

Amino acid	*mg/100 ml of plasma*								
	Trimethylsilyl			*N-TFA butyl esters*			*Ion-exchange chromatography*		
			Av.			*Av.*			*Av.*
Alanine	1.46	1.43	1.45	1.51	1.45	1.48	1.49	1.53	1.52
Valine	2.61	2.67	2.64	2.66	2.72	2.69	2.67	2.79	2.73
Leucine	1.71	1.74	1.73	1.81	1.83	1.82	1.79	1.81	1.80
Isoleucine	1.21	1.19	1.20	1.29	1.31	1.27	1.21	1.20	1.21
Glycine	1.37	1.48	1.43	1.38	1.44	1.42	1.41	1.53	1.47
Proline	0.97	0.88	0.93	0.91	0.94	0.93	0.91	0.95	0.93
Serine	0.74	0.78	0.76	0.80	0.81	0.80	0.80	0.81	0.80
Threonine	0.71	0.70	0.71	0.71	0.73	0.72	0.75	0.73	0.74
Hydroxyproline	0.21	0.22	0.22	0.22	0.23	0.23	0.26	0.27	0.27
Aspartic acid	0.08	0.11	0.10	0.32[a]	0.32[a]	0.32[a]	0.10	0.10	0.10
Methionine	0.22	0.19	0.21	0.22	0.22	0.22	0.24	0.22	0.23
Glutamic acid	2.41	2.26	2.34	4.07[a]	4.21[a]	4.14[a]	2.37	2.42	2.40
Phenylalanine	0.72	0.76	0.74	0.71	0.74	0.73	0.70	0.71	0.70
Arginine	2.21[b]	2.07[b]	2.14[b]	1.38	1.40	1.39	1.44	1.47	1.46
Lysine	1.36	1.32	1.34	1.43	1.45	1.44	1.23	1.30	1.27
Tyrosine	0.54	0.59	0.57	0.63	0.61	0.62	0.59	0.60	0.59
Histidine	0.69	0.75	0.72	0.74	0.76	0.75	0.78	0.79	0.79
Tryptophan	0.31	0.29	0.30	0.29	0.27	0.28	0.23	0.24	0.23
Cystine	—	—	—	trace	trace	trace	trace	trace	trace
Ornithine	—[b]	—[b]	—[b]	1.04	1.10	1.07	1.08	1.09	1.09
Totals	19.53	19.43	19.48	22.13	22.53	22.33	20.01	20.56	20.29

[a] Values include aspartic acid plus asparagine, and glutamic acid plus glutamine.
[b] Values include arginine and ornithine.

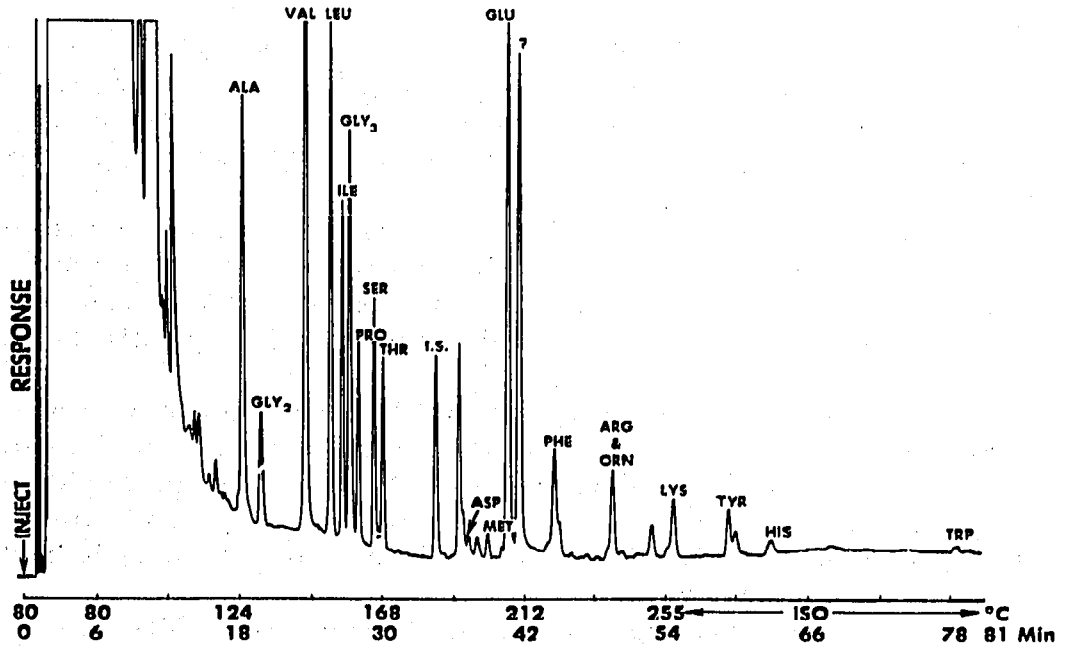

Fig. 8. Gas–liquid analysis of TMS amino acids of bovine blood plasma. Sample: 10 ml of plasma, cation-exchange cleaned. Derivatization: 2 ml, BSTFA–CH_3CN (1:1), 150° for 2.5 h. Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm I.D. glass. Conditions: injector, 275°; detector, 300°; initial temperature, 80°; 6 min initial hold, 3.5°/min to 255°; carrier gas N_2, 17 ml/min, I.S. = decanoic acid.

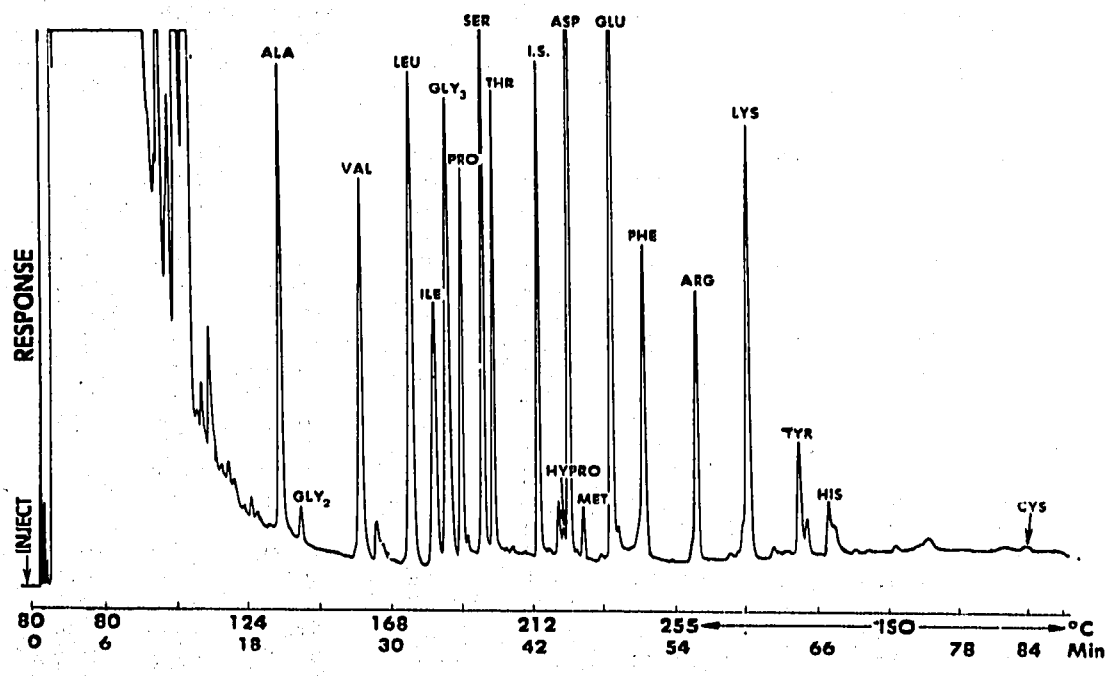

Fig. 9. Gas–liquid analysis of TMS amino acids of soybean meal. Sample: 4 mg of cation-exchange cleaned soybean meal hydrolysate. Derivatization: 2 ml, BSTFA–CH_3CN (1:1); 150° for 2.5 h. Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 6 m × 2 mm I.D. glass. Conditions: injector, 275°; detector, 300°; initial temperature, 80°; 6 min initial hold, 3.5°/min to 255°: carrier gas N_2, 17 ml/min, I.S. = decanoic acid.

TABLE IX

AMINO ACID ANALYSIS OF SOYBEAN MEAL

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

Amino acid	*Trimethylsilyl*			*N-TFA butyl esters*			*Ion-exchange chromatography*		
			Av.			*Av.*			*Av.*
Alanine	2.10	2.09	2.10	2.18	2.16	2.17	2.10	2.11	2.10
Valine	2.22	2.29	2.25	2.35	2.30	2.33	2.37	2.50	2.43
Leucine	3.37	3.42	3.40	3.45	3.44	3.45	3.50	3.62	3.56
Isoleucine	2.03	2.09	2.06	2.13	2.09	2.11	2.09	2.20	2.14
Glycine	1.92	1.89	1.90	2.00	2.05	2.03	1.94	1.96	1.95
Proline	2.61	2.79	2.70	2.82	2.90	2.86	2.73	2.55	2.64
Serine	2.84	2.89	2.87	2.74	2.83	2.79	2.75	2.62	2.68
Threonine	1.76	1.81	1.79	1.85	1.89	1.87	1.86	1.82	1.84
Hydroxyproline	0.04	0.06	0.05	0.09	0.10	0.10	trace	trace	trace
Aspartic acid	5.24	5.37	5.31	5.32	5.14	5.23	5.31	5.16	5.24
Methionine	0.42	0.39	0.40	0.44	0.51	0.48	0.35	0.37	0.36
Glutamic acid	8.24	7.79	8.02	8.18	7.82	8.05	8.22	7.88	8.05
Phenylalanine	2.35	2.41	2.38	2.34	2.33	2.34	2.32	2.50	2.41
Arginine	2.85	2.98	2.92	3.48	2.88	3.18	3.19	3.44	3.32
Lysine	2.57	2.74	2.66	3.03	2.38	2.96	2.81	2.79	2.80
Tyrosine	1.27	1.34	1.30	1.38	1.29	1.34	1.31	1.43	1.37
Histidine	—	—	—	1.62	1.57	1.59	1.44	1.54	1.49
Tryptophan[a]	—	—	—	—	—	—	—	—	—
Cystine	0.21	0.22	0.22	0.26	0.26	0.26	0.23	0.30	0.26
Totals	42.89	43.35	43.12	45.73	44.34	45.04	44.56	44.82	44.69

[a] Destroyed during hydrolysis.

trifluoroacetyl butyl ester derivatives of a soybean meal hydrolysate is presented in Table IX. Fig. 9 presents the corresponding chromatogram for the TMS derivatives. The results of these analyses are seen to be in excellent agreement.

Chromatographic separation. Short column

In analyses of most biological samples, the nearly baseline separations, as presented in Fig. 1, are not required to obtain a quantitative analysis for amino acids as the N-TMS-TMS ester derivatives. The 6 m column used in most of this work has several disadvantages associated with all small bore long columns *i.e.*, high back pressure, difficulty in obtaining a good packed column, time required in packing, and column expense. A separation sufficient for the quantitative analysis of amino acids in most biological samples without the disadvantages of the long 6 m column is presented in Fig. 10. This chromatogram was obtained with a 2 m × 1.6 mm I.D. glass column containing 10 w/w% OV-11 on 100/120 mesh Supelcoport.

Derivatization and chromatography of other amino acids

Since over two hundred amino acids have been found to occur in nature, it was desired to show that BSTFA can be used for the analysis of many non-protein amino acids. Table X gives the relative retention time and $R.W.R._{\text{a.a./fluorene}}$ for twelve non-protein amino acids derivatized at 150° for 15 min and chromatographed on a

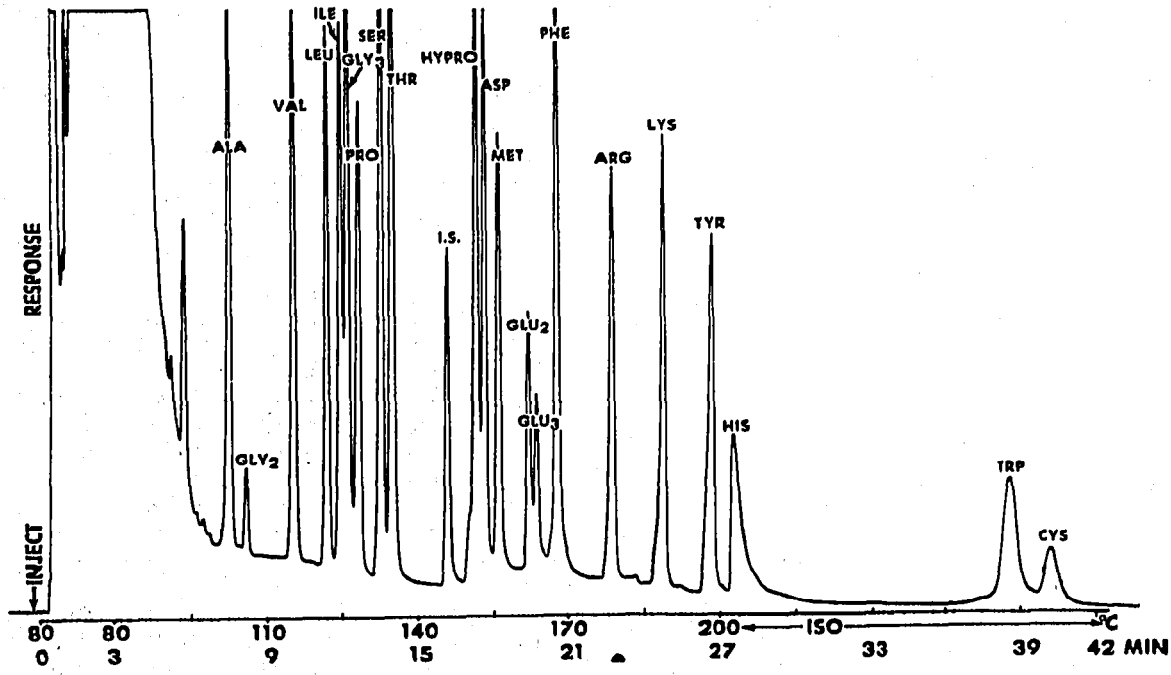

Fig. 10. Gas–liquid chromatogram of TMS amino acid derivatives. Sample: 2 mg of total amino acids. Derivatization, 1 ml, BSTFA–CH_3CN (1:1); 150° for 2.5 h. Injected: 5 μl (0.5 μg of each amino acid). Column: 10 w/w% OV-11 on 100/120 mesh Supelcoport, 2 m × 1.6 mm I.D. glass. Conditions: injector, 275°; detector, 300°; initial temperature, 80°; 3 min hold, 5°/min to 210°; carrier gas N_2, 17 ml/min, I.S. = decanoic acid.

2 m × 4 mm I.D. 10 w/w% OV-7 on 100/120 mesh Supelcoport column. Temperatures of 50°, 100°, and 150°, were used in the derivatization experiments. All of the selected non-protein amino acids except for penacillamine disulfide, ornithine, and creatinine, can be derivatized at 50° for 30 min with *R.W.R.* values as given in

TABLE X

RELATIVE WEIGHT RESPONSE AND RELATIVE RETENTION TIME OF SELECTED NON-PROTEIN AMINO ACIDS

Amino acid	*Relative retention time*[a]	*R.W.R.a.a./fluorene*
α-Aminobutyric acid	0.49	0.85
β-Aminobutyric acid	0.53	0.81
Norvaline	0.55	1.22
Methionine sulfoxide	0.72	0.56
γ-Aminobutyric acid	0.74	0.91
Norleucine	0.74	1.18
Creatinine	0.93	0.72
Cysteic acid	1.05	0.74
Penacillamine	1.07	1.27
Ornithine	1.07	0.83
Methionine sulfone	1.12	0.62
Penacillamine disulfide	1.47	0.71

[a] 2 m × 4 mm I.D. glass column, 10 w/w% OV-7 on 100/120 mesh Supelcoport. Silylation at 150° for 15 min in acetonitrile.

Table X. However, it was necessary to derivatize ornithine, creatinine, and penacillamine disulfide at 150° for 15 min to obtain the $R.W.R._{a.a./fluorene}$ values given in Table X.

Preliminary investigations on some common substances in biological samples have indicated that asparagine, glutamine, and glucosamine can be derivatized and separated by the techniques used for the twenty protein amino acids. Asparagine gave two chromatographic peaks, with the first peak eluting between methionine and glutamic acid, and the second peak eluting immediately after phenylalanine. Glutamine gave one chromatographic peak which eluted shortly after arginine. Glucosamine resulted in two chromatographic peaks with both eluting between arginine and lysine. Both peaks for glucosamine were well resolved from glutamine. Although further study is necessary before these compounds can be analyzed quantitatively, these compounds appear amenable to analysis by this method. Investigations on these and other non-protein amino acids will be the subject of another paper.

The TMS method for the protein amino acids is complementary to the N-trifluoroacetyl (N-TFA) butyl ester technique[15,16]. This is illustrated in Tables VIII and IX. With the TMS derivatives one can obtain analyses for aspartic acid and glutamic acid alone, while the N-TFA butyl ester technique results in values which include asparagine and glutamine, due to their hydrolysis during derivatization. The N-TFA butyl ester derivative provides individual values for ornithine and arginine whereas the TMS derivative gives a combined value due to simultaneous elution.

CONCLUSIONS

These experiments have conclusively demonstrated that the twenty protein and many non-protein amino acids can be quantitatively determined by GLC as their TMS derivatives. This method can be used for the most complex biological samples with appropriate prior cleanup using cation and anion exchange procedures. Important advantages are simplicity and speed, as the derivatization involves only the addition of reagents with no transfers, and the chromatographic separation can be made on a single column in 60 to 80 min. The column is glass, 6 m × 4 mm I.D., 10 w/w% OV-11 on 100/120 mesh Supelcoport. If desired, a short column can be used on which lysine, phenylalanine, histidine, tryptophan, and cystine are separated in 20 min. The column is 2 m × 4 mm I.D., glass, 10 w/w% OV-7 on 100/120 mesh Supelcoport. This analytical method has been demonstrated to be complementary to the N-trifluoroacetyl butyl ester technique for the GLC analysis of protein amino acids also developed by this laboratory.

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