CHROM. 4995

TRIMETHYLSILYLATION OF AMINO ACIDS*

EFFECT OF SOLVENTS ON DERIVATIZATION USING BIS(TRIMETHYLSILYL)TRIFLUOROACETAMIDE

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

(Received August 17th, 1970)

SUMMARY

The number of chromatographic peaks for the TMS derivatives of glycine and arginine are determined by the polarity of the solvent. With hexane, methylene chloride, chloroform, and 1,2-dichloroethane, one peak is obtained for glycine (GLY_2), and two peaks (GLY_2 and GLY_3) in six other more polar solvents. Arginine gives no peak in the four less polar solvents studied and one peak in the other six more polar solvents.

Preliminary work is reported on chromatography using a 6 m × 2 mm I.D. glass column of 10 w/w% OV-11 on 100/120 mesh Supelcoport of which good resolution was obtained for the TMS derivatives of nineteen of the protein amino acids. More detailed chromatographic studies will be the subject of a separate paper.

INTRODUCTION

Since the introduction of the trimethylsilyl (TMS) derivatives of the amino acids by RÜHLMANN AND GIESECKE[1], efforts have been made by several groups of researchers in attempts to use these derivatives for the quantitative gas–liquid chromatographic (GLC) analysis of the twenty protein amino acids. The primary interest in the TMS derivative is the one-step derivatization procedure, whereas almost all other derivatives are formed by two or more reaction steps. RÜHLMANN AND GIESECKE used hexamethyldisilazane and trimethylchlorosilane (TMCS) to obtain derivatives for most of the protein amino acids. SMITH *et al.*[2,3] made a study of the optimum silylation conditions for leucine, serine, and aspartic acid. They concluded that trimethylsilyldiethylamine with some kind of catalyst was the best silylation reagent. Trimethylsilyldimethylamine recently has been claimed to be more volatile

* Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 6061. Approved by the Director. Supported in part by grants from the National Aeronautics and Space Administration (NGR 26-004-011) and the National Science Foundation (GB 7182).

** Professor. Experiment Station Chemical Laboratories.

*** Experimental data taken in part from master's research, University of Missouri.

and was recommended for these reasons[4]. N-Trimethylsilyl-N-methyl-acetamide has been recommended by BIRKOFER AND DONIKE[5]. KLEBE *et al.*[6] used bis(trimethylsilyl)acetamide (BSA) to obtain sharp single peaks for all the protein amino acids except arginine, which showed indications of decomposition on the column. However, they were unable to separate the derivatives of glycine and alanine from BSA on an SE-30 column. The introduction of bis(trimethylsilyl)trifluoroacetamide (BSTFA) by STALLING *et al.*[7] has solved the problem of separation of the TMS derivatives of glycine and alanine from the reagents and reaction products. Then, GEHRKE *et al.*[8,9] published a comprehensive method for the GLC analysis of all twenty protein amino acids as their TMS derivatives using BSTFA as the silylating agent, but they reported that problems still existed in the analysis of biological fluids such as urine.

BERGSTRÖM *et al.*[10] recently reported on the trimethylsilylation of amino acids. These workers used BSTFA with and without solvent at 125° for 15 min to obtain chromatographic peaks for eighteen amino acids. They also reported on the mass spectra of the two derivatives of glycine and lysine. The results of the mass spectra are in agreement with the structural assignment given by GEHRKE and coworkers[8,9].

GEHRKE and coworkers report that urine samples containing large amounts of glycine presented difficulties in obtaining a single peak for glycine. Both the di-trimethylsilyl (GLY_2) derivative and the tri-trimethylsilyl (GLY_3, *ca.* 10%) derivatives were obtained when the samples were derivatized at 135° for both 10 and 15 min. Because of the large quantity of glycine in the urine samples, the GLY_3 peak interfered with the resolution of TMS isoleucine and TMS proline. In preliminary work on the TMS derivative of glycine by the present authors, it was observed that only the first peak for glycine (GLY_2) was obtained when using methylene chloride as a solvent instead of acetonitrile. This study was undertaken to investigate the effect of different solvents on silylation and reports on improvements in the chromatographic separation, thus permitting the analysis of biological fluids such as urine.

EXPERIMENTAL

Reagents and materials

Acetonitrile, hexane, and chloroform were obtained from Mallinkrodt Chemical Works, St. Louis, Mo., and were of "Nanograde" purity. Methylene chloride was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and was analytical reagent grade. Pyridine, 1,2-dichloroethane, dimethylformamide, tetrahydrofuran, and triethylamine were obtained from Distillation Products Industries, Rochester 3, N.Y. All solvents were dried over calcium chloride and redistilled before use.

The BSTFA (Regisil) was obtained from Regis Chemical Company, Chicago, Ill.

The OV-7, OV-11, and OV-22 liquid phases and solid support, Supelcoport, 100/120 mesh, were obtained from Supelco, Inc., Bellefonte, Pa.

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

Equipment

A Micro-Tek Model MT-220 gas chromatograph with a four-column oven bath, two dual-channel electrometers and four flame ionization detectors were used in this study. A Varian Model 30 recorder was used for the chart presentation.

Chromatographic column

The chromatography column was a mixed phase consisting of 3 w/w% OV-22 and 6 w/w% OV-7 on 100/120 mesh Supelcoport in a 2 m × 4 mm I.D. glass column. Also used was 10% OV-11 on Supelcoport in a glass column 6 m × 2 mm I.D.

Derivatization method

Two milliliters of a stock solution containing 0.1 mg/ml of each amino acid were pipetted into a Corning No. 9826 1.6 cm × 7.5 cm reaction tube and dried under a stream of dry filtered nitrogen at 75°. One milliliter of methylene chloride was added and evaporated under nitrogen to azeotrope any remaining water. Then 1 ml of BSTFA and 1 ml of acetonitrile were added to the tube. The sample tube was closed with a teflon-lined cap and heated at different temperatures and times in a constant-temperature oil bath. For the solvent study, the only change made was to substitute the appropriate solvent for acetonitrile. Also, in some experiments, 1% TMCS in BSTFA was substituted for the BSTFA.

RESULTS

Table I presents a summary of the solvents used in this research and their results. No quantitation was attempted using these solvents. In these experiments,

TABLE I

SOLVENT EFFECTS ON TMS DERIVATIZATION OF PROTEIN AMINO ACIDS

Solvent	*No. of glycine peaks*	*No. of arginine peaks*
Acetonitrile	2	1
Dimethylformamide	2	1
Pyridine	2	1
Triethylamine	2	1
Tetrahydrofuran	2	1
10% acetonitrile in methylene chloride	1	0
5% acetonitrile in methylene chloride	1	0
Hexane	1	0
Methylene chloride	1	0
Chloroform	1	0
1,2-Dichloroethane	1	0

essentially the same results were obtained on a qualitative basis as reported by GEHRKE *et al.*[8], as illustrated in Figs. 1 and 2. GEHRKE and coworkers reported two peaks for glycine and one peak for arginine using acetonitrile as solvent. In this study, using methylene chloride, hexane, chloroform, and 1,2-dichloroethane, it was observed that glycine produced only one peak, as illustrated in Figs. 3 and 4, whereas two peaks were formed in acetonitrile, dimethylformamide, pyridine, triethylamine, and tetrahydrofuran. The number of peaks for glycine derivatized in the non-polar solvents was not affected by the time or temperature, as times from 15 min to 6 h, and temperatures from 50° to 150° were used. Also, arginine under the same experimental conditions is either not derivatized, or decomposed, as no peak was obtained. In these

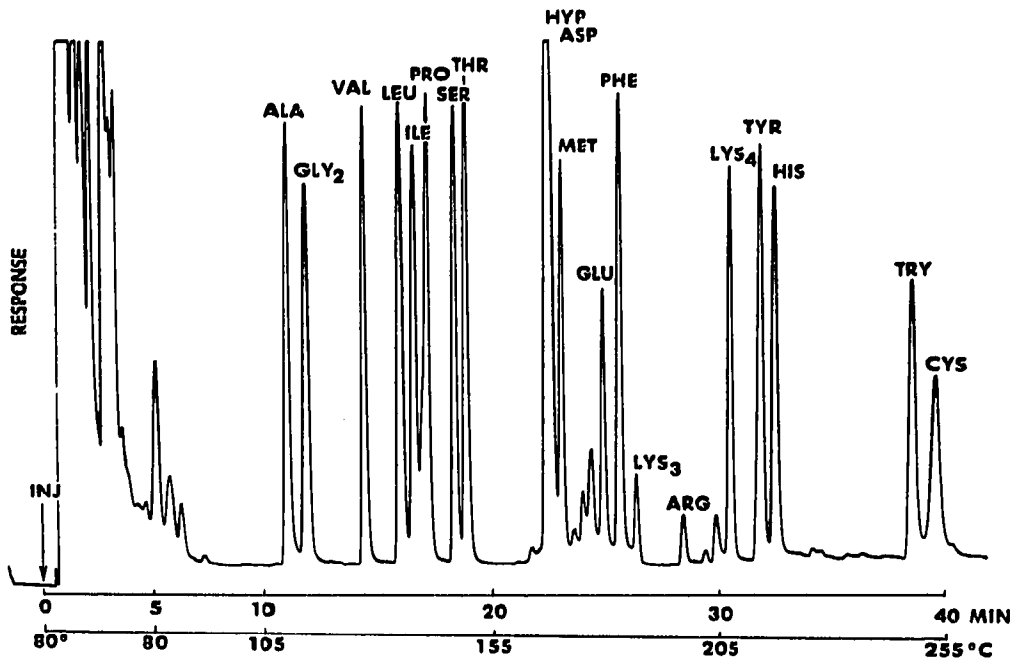

Fig. 1. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_3CN–BSTFA (1:1) after closed-tube silylation at 135° for 15 min. Conditions: Column, mixed liquid phase 6.0 w/w% OV-7 and 3.0 w/w% OV-22 on 100/120 mesh Supelcoport, 2 m × 4 mm I.D., glass. Temperature, programmed, initial temperature 80°, 5 min hold, then 5°/min. Volume injected, 5 μl.

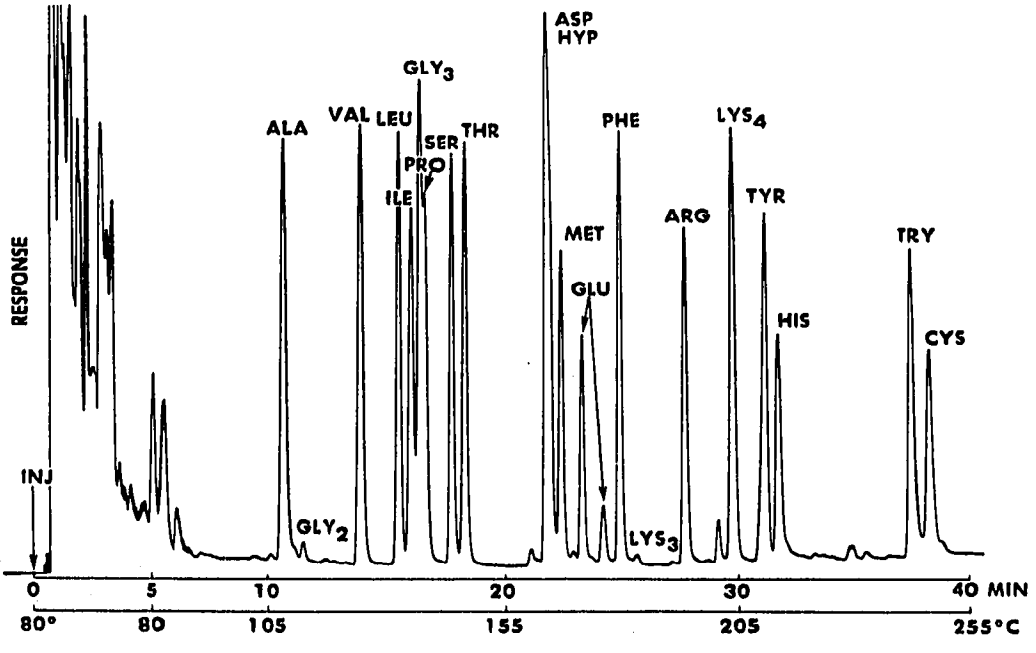

Fig. 2. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_3CN–BSTFA (1:1) after closed-tube silylation at 135° for 4 h. Conditions, see the legend to Fig. 1.

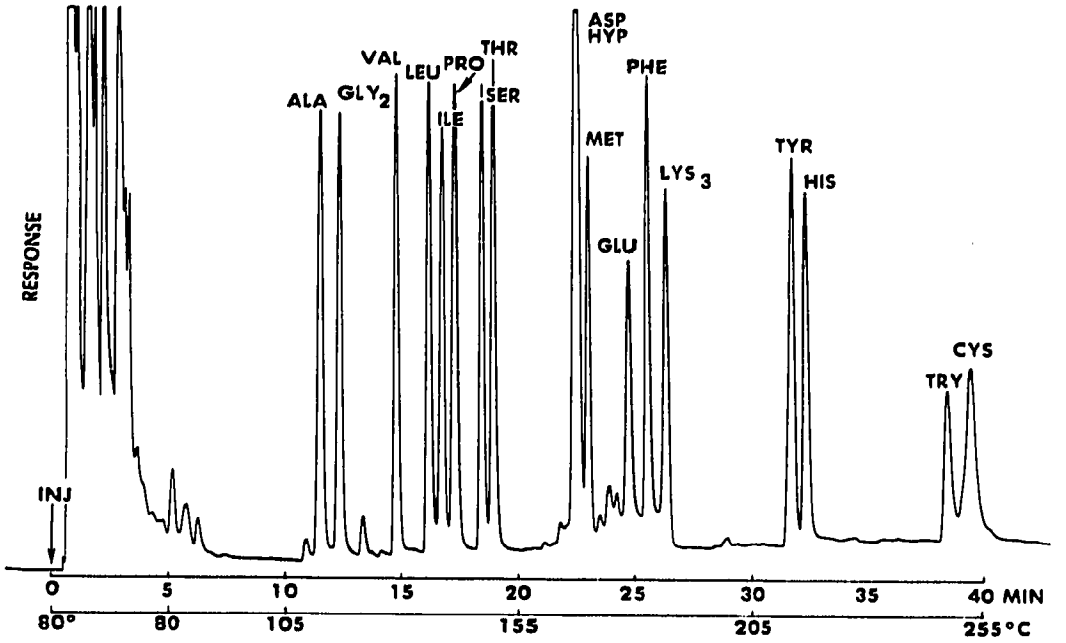

Fig. 3. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_2Cl_2–BSTFA 1:1) after closed-tube silylation at 135° for 15 min. Conditions, see the legend to Fig. 1.

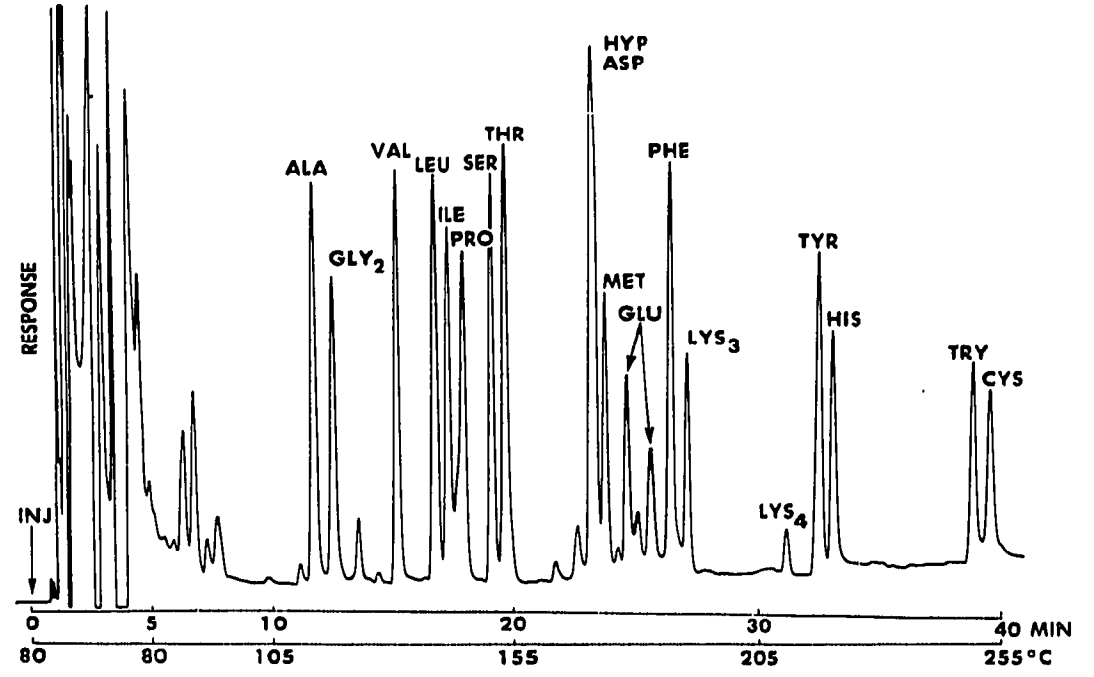

Fig. 4. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_2Cl_2–BSTFA (1:1) after closed-tube silylation at 135° for 4 h. Conditions, see the legend to Fig. 1. Each peak represents *ca.* 500 ng.

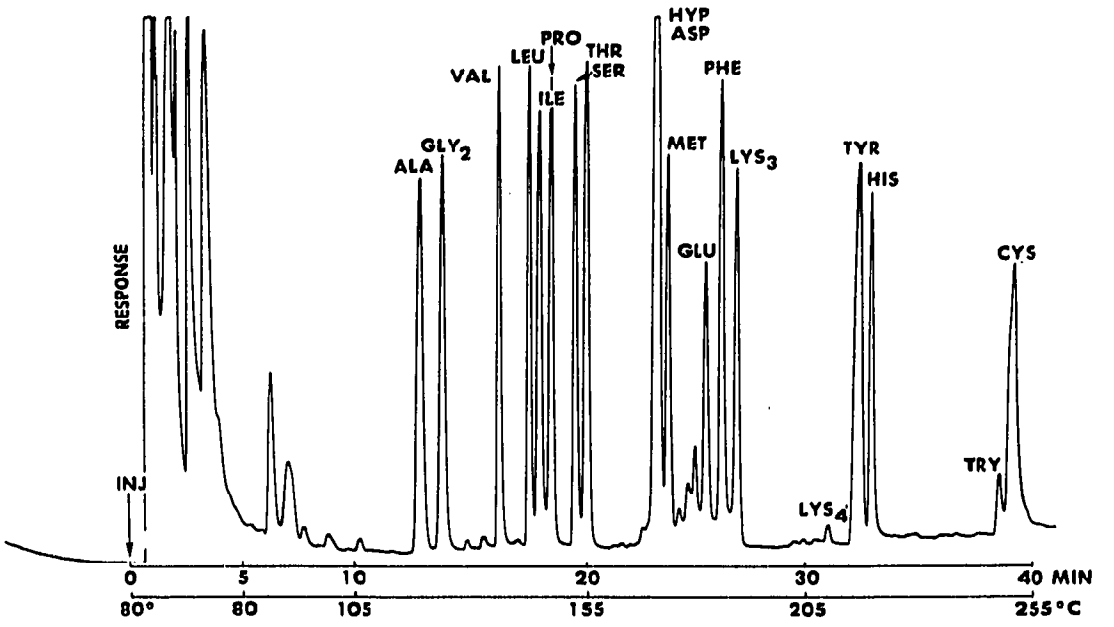

Fig. 5. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_2Cl_2–1% TMCS in BSTFA (1:1) after closed-tube silylation at 135° for 15 min. Conditions, see the legend to Fig. 1.

non-polar solvents, one notes two peaks for glutamic acid, mostly LYS_3 instead of LYS_4, no peak for arginine, and nearly all GLY_2.

CHAMBAZ *et al.*[11–13] has recently reported that the use of 1% TMCS has a catalytic effect on the silylation of steroids using BSA. Regis Chemical Company[14] has suggested

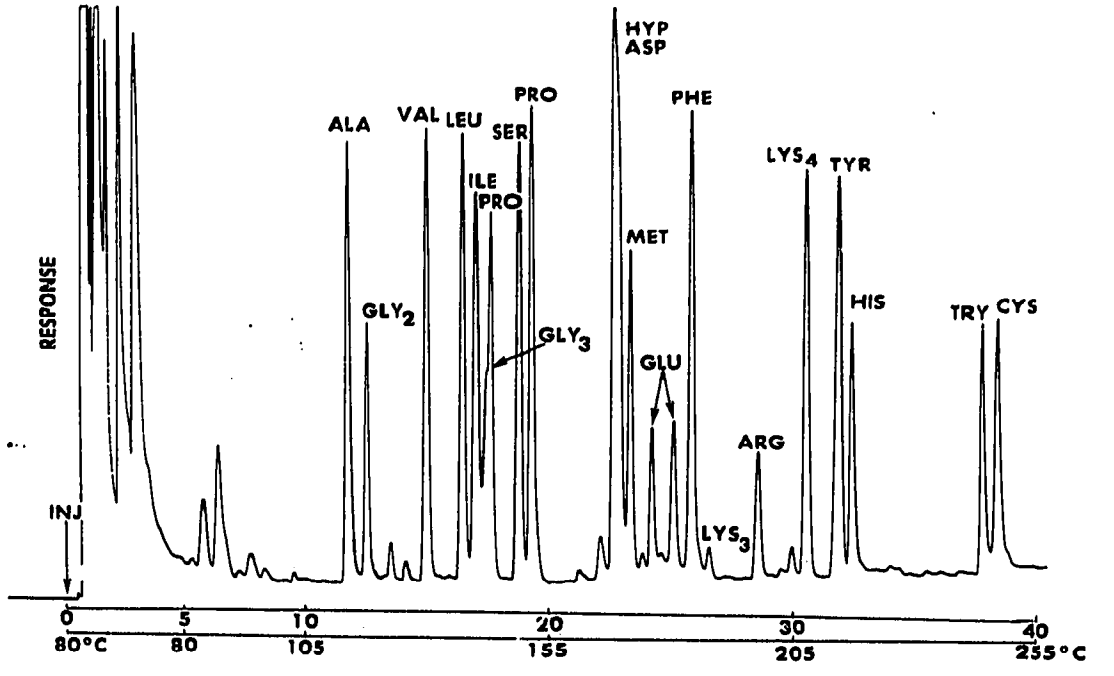

Fig. 6. Chromatogram obtained on GLC of 0.2 mg of each amino acid in 2.0 ml CH_2Cl_2–1% TMCS in BSTFA (1:1) after closed-tube silylation at 135° for 4 h. Conditions, see the legend to Fig. 1.

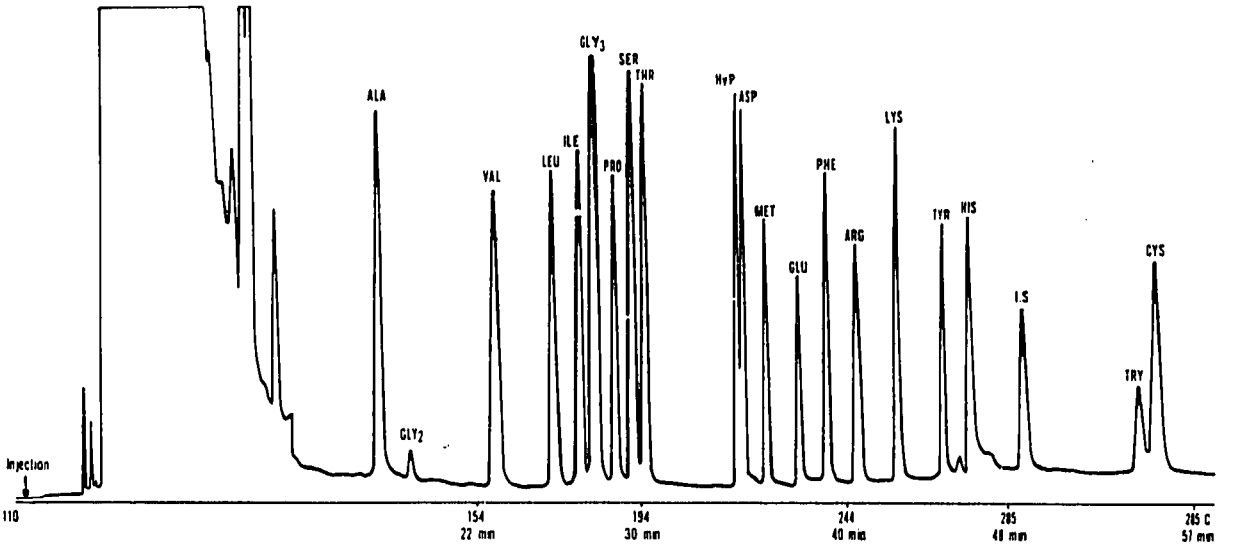

Fig. 7. Chromatogram obtained on GLC of 1.0 μg of each amino acid (2.0 mg total amino acids) in 1.0 ml BSTFA–CH_3CN (1:1) after closed-tube silylation at 150° for 2.5 h. Conditions: Column, 10% OV-11 on Supelcoport 100/120 mesh, 6 m × 2 mm I.D., glass. Injector temperature, 275°. Detector temperature, 300°. Column temperature, programmed, initial temperature 110°, 2°/min for 22 min, then 5°/min up to 285°. Nitrogen flow rate, 20 ml/min. Internal standard, phenanthrene.

the use of 1% TMCS with BSTFA. Figs. 5 and 6 show the effect of the use of 1% TMCS with BSTFA in methylene chloride. The use of 1% TMCS as a catalyst results in a more powerful silylating agent as there is a larger amount of GLY_3, LYS_4, and an arginine peak on silylation at 135° for 4 h (Fig. 6). As shown in Fig. 5, silylation under these conditions for 15 min is incomplete. No differences were noted with 1% TMCS as catalyst in a polar solvent, acetonitrile, from the use of BSTFA in acetonitrile.

It appears that the derivatization of glycine, arginine, lysine, and glutamic acid involves the polarity of the solvent, with polar solvents giving two peaks for glycine and one peak for arginine. In non-polar solvents only the first chromatographic peak for glycine and no peaks for arginine were obtained. The explanation for not obtaining a peak for arginine is probably due to solubility of arginine in the solvent, or a difference in the silylating strength of BSTFA in various polarity solvents.

There is a need to expand the earlier work of GEHRKE and coworkers, especially in the area of chromatographic resolution. The results of preliminary studies are given in Fig. 7. This chromatogram shows excellent resolution for nineteen amino acids. Further work is in progress to investigate the chromatography on this column, a 6 m × 2 mm I.D. 10 w/w% OV-11 on 100/120 mesh Supelcoport.

In the analysis of biological fluids containing large amounts of glycine it may be necessary to derivatize the sample with two different solvents to avoid interference of the GLY_3 peak with isoleucine and proline.

REFERENCES

1 K. RÜHLMANN AND W. GIESECKE, *Angew. Chem.*, 73 (1961) 113.
2 E. D. SMITH AND H. SHEPPARD, JR., *Nature*, 208 (1965) 878.
3 P. S. MASON AND E. D. SMITH, *J. Gas Chromatog.*, 4 (1966) 398.
4 *Chromatography/Lipids*, Supelco, Inc., Bellefonte, Pa., 1 (1967) 3.

5 L. BIRKOFER AND M. DONIKE, *J. Chromatog.*, 26 (1967) 270.
6 J. KLEBE, H. FINKBEINER AND D. WHITE, *J. Am. Chem. Soc.*, 88 (1966) 3390.
7 D. L. STALLING, C. W. GEHRKE AND R. W. ZUMWALT, *Biochem. Biophys. Res. Commun.*, 31 (1968) 616.
8 C. W. GEHRKE, H. NAKAMOTO AND R. W. ZUMWALT, *J. Chromatog.*, 45 (1969) 24.
9 H. NAKAMOTO, *Master's Thesis*, University of Missouri, June, 1969.
10 K. BERGSTRÖM, J. GÜRTLER AND R. BLOMSTRAND, *Anal. Biochem.*, 34 (1970) 74.
11 E. CHAMBAZ AND E. C. HORNING, *Anal. Letters*, 1 (1967) 201.
12 E. CHAMBAZ AND E. C. HORNING, *Anal. Biochem.*, 30 (1967) 7.
13 E. CHAMBAZ, G. MARME AND E. C. HORNING, *Anal. Letters*, 1 (1968) 749.
14 *Gas Chromatography Catalog*, Regis Chemical Company, Chicago, Ill., 1970, p. 21.