# GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF N-TRIFLUORO-ACETYL AMINO ACID ISOPROPYL ESTERS

An Abstract of

A Thesis

Presented to the Department of Biophysical Sciences

College of Arts and Sciences

University of Houston

In partial fulfillment

of the requirements for the degree

Master of Science

by

Achim Seiler

December 1972

# ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. J. Oró for his guidance, patience, and support during this course of study. The contributions from discussions with members of the biomolecular analysis group, Dr. Parr's group, and Dr. Zlatkis' group are also acknowledged.

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## ABSTRACT

All primary α-amino acids detected in the Murchison Meteorite were also found in Miller's recent prebiotic synthesis of amino acids by electric discharge. A procedure was established that allows the separation and ready identification of these amino acids by gas chromatography-mass spectrometry. The retention times of the amino acids as their N-trifluoro-acetyl (N-TFA) isopropyl ester derivatives were determined in methylene units in comparison with a standard of aliphatic hydrocarbons on a SF-96 stainless steel needle stock. The mass spectra of the derivatized amino acids were taken and standardized. These standards have been recorded on magnetic tape and will be used for the computerized identification of mass spectra of N-TFA amino acid isopropyl esters from extraterrestrial or prebiotic samples to be investigated.

The applicability of the method was tested on samples from a polymerization experiment of hydrogencyanide received from J. P. Ferris. Five protein and two non-protein amino acids were isolated, separated, and identified by gas chromatography in combination with mass spectrometry.

## Abbreviations of Amino Acids Chosen for Study

| | |
|---|---|
| ala | Alanine |
| β-ala | β-Alanine |
| aile | Alloisoleucine |
| athr | Allothreonine |
| α-aba | α-Aminobutyric acid |
| β-aba | β-Aminobutyric acid |
| γ-aba | γ-Aminobutyric acid |
| α-aib | α-Aminoisobutyric acid |
| β-aib | β-Aminoisolbutyric acid |
| arg | Arginine |
| asp | Aspartic acid |
| $cys_2$ | Cystine |
| cys | Cysteine |
| dab | 2,4-Diaminobutyric acid |
| dap | Diaminopropionic acid |
| das | Diaminosuccinic acid |
| eth | Ethionine |
| glu | Glutamic acid |
| gly | Glycine |
| his | Histidine |
| hyp | Hydroxyproline |
| ile | Isoleucine |
| iser | Isoserine |

| | |
|---|---|
| ival | Isovaline |
| leu | Leucine |
| lys | Lysine |
| met | Methionine |
| m-β-ala | N-Methyl-β-alanine |
| m-γ-aba | N-Methyl-γ-aminobutyric acid |
| m-leu | N-Methylleucine |
| m-val | N-Methylvaline |
| nleu | Norleucine |
| nval | Norvaline |
| phe | Phenylalanine |
| ppa | Pipecolic acid |
| pro | Proline |
| sar | Sarcosine |
| thr | Threonine |
| try | Tryptophan |
| tyr | Tyrosine |
| val | Valine |

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# I.

# INTRODUCTION

# I. INTRODUCTION

Derivatives of amino acids and their gas chromatography:

Derivatives of amino acids can be separated in less than one hour on appropriate GC columns and the amount of amino acid required is usually in the microgram range. Thus, the method is especially suitable for trace analysis and can be used in the determination of small amounts of amino acids in extraterrestrial, natural or synthetic samples. However, gas chromatography cannot provide a characterization of the molecular structure of amino acids in unknown mixtures. On the other hand, mass spectrometry offers a means of identification and structure elucidation of organic compounds. The combination of both techniques, gas chromatography and mass spectrometry is uited for separation and identification of amino acids. For quantitative investigation of amino acids, packed GC columns seem to be more suitable than capillary columns. Capillary columns however provide a greater separation efficiency in shorter time and are preferred for qualitative analytical investigations, especially when a combined gas chromatograph-mass spectrometer is used.

The approximately twenty amino acids commonly found in proteins, as well as the nonprotein amino acids, formed by prebiotic synthesis or detected in meteorites, have very varied chemical structures. Due to the different

reactivities of the chemical groupings, the choice of the volatile derivatives presents a problem.

In early attempts the amino acids were converted to aldehydes and these products chromatographed. The aldehydes formed by ninhydrin oxidation in a microreactor at 130°C were separated by Zlatkis and Oro' (1) and then hydrocracked, so that they could be detected and analyzed as methane.

The underivatized amino acid is not suitable for gas chromatography due to the twitter ionic character of the molecule. Removal of the amino group by deamination is not easy to achieve for all the amino acids. The replacement of the amino group by a chloro group proved to be quantitative and GC analysis of α-chloro acid methyl esters gave good separations. However, not all the amino acids could be derivatized (2). It was also attempted to separate amino acids in their free base form as methyl esters. These derivatives are volatile. However, cys, his, tyr and try could not be chromatographed (3). Bayer was the first one to show that N-TFA amino acid methyl esters are suitable for gas chromatography. It was found that derivatives in which both the amino and the carboxy group of the amino acid are derivatized yielded better results.

For double derivatization, two chemical reactions usually have to be carried out and high yields at each step

are required. All double derivatives have the carboxy group esterified. Methyl esters will give the most volatile derivative. Amino acid hydrochlorides are good soluble in methanol HCl and esterification is easy to achieve. For esterification the amino acid is either suspended in alcohol and dry hydrogen chloride bubbled through the mixture or the amino acid is dissolved in an alcoholic solution of hydrogen chloride. Instead of hydrochloric acid, concentrated sulfuric acid has been used as a catalyst. Also Lewis acids like thionyl chloride or bortrifluoride may be used (4).

In order to overcome solubility problems of certain amino acids the compound can be dissolved in little anhydrous trifluoroacetic acid before adding the alcohol-HCl solution (5). Apart from the solubility problem, higher alcohols are less reactive than methanol. Thus, instead of direct esterification it is preferable in some cases to prepare the methyl esters first and then to transesterify them with the higher alcohol. This transesterification is especially necessary for arg, lys, tyr, try and cys. The method was first adopted by Gehrke (6) in the preparation of n-butyl esters.

The most common way to derivatize the amino group is acylation. Commonly used is trifluoroacetic acid anhydride (TFAA) as the acylating agent, because the resulting

derivative does not appreciably reduce the volatility of the amino acid ester. The TFAA can be directly reacted with the amino acid ester hydrochloride without isolation of the free base.

The nonfluorinated acyl groups have also been used in amino acids for gas chromatography. But the N-acetyl, N-formyl or N-propionyl group are less volatile than the TFA group and the chromatographic peaks are less efficient.

In some applications, it was tried to match the kind of derivative to the detector. For example, the N-TFA group was found not to be suitable for electron capture detector; however, the O-TFA group is strongly electron capturing. The higher perfluorinated alkyl groups like pentafluoro-propionyl (PFP) and heptafluorobutyryl (HFB) groups are highly electron capturing. PFP and HFB amino acid n-butyl ester show shorter retention times than the corresponding TFA amino acid n-butyl esters. Pollock (7) found the reaction conditions for the preparation of PFP and HFB derivatives to be the same as for TFA derivatives. The separation of these derivatives appeared to be good.

Almost all of the proposed derivatives require a two or even three step preparation procedure. To avoid so many manipulations of the sample and losses during derivatization, especially in quantitative analysis a single step procedure would be the ideal approach.

A one step procedure would be the permethylation of the amino acid. This technique is used in the analysis of peptides. The volatility of the permethylated peptide allows for sequence analysis by mass spectrometry. A similar approach for the one-step derivatization procedure of amino acids was taken by B. C. Pettitt and J. E. Stouffer (8). In this procedure, the amino acid is reacted with 2-bromopropane to yield the N, O-diisopropyl derivative. With the exception of arginine, all the protein amino acids yielded the expected isopropyl derivatives. The mass spectrometric fragmentation patterns of this type of derivative do not seem to be as distinctive and characteristic as obtained from the N-TFA O-isopropyl derivatives.

The most detailed work on the analysis of amino acids has been reported by Gehrke. He developed a technique for quantitatively analyzing the protein amino acids as their volatile N-TFA n-butyl esters (9) derivatives. Furthermore, the complete gas chromatographic resolution of the protein amino acids on a single substrate has been difficult to achieve. Even after ethylene glycol adipate (EGA) was introduced as a stationary phase, the quantitative elution of arg, his, $cys_2$ and try could not be achieved (10). A dual column system consisting of EGA and mixed siloxane phases OV-17 and OV-210 is now used to simultaneously analyze and separate the 20 protein amino acids when they

are derivatized as N-TFA alkyl esters (11).

The use of two columns for the separation of the twenty protein amino acid cannot be the final solution even though there are some advantages over a single column system, e.g. better resolution, cross confirmation for some amino acids and easier identification of the eluted compounds.

The superior solution, the successful separation of the twenty protein amino acids on a single column, was finally achieved by Moss et al (12). This research group used OV-1 as the liquid phase and observed separation of 18 of the 20 protein amino acid when prepared as N-TFA n-propyl esters. The separation of the twenty amino acids was achieved by substituting the N-TFA group by the N-HFB group which slightly decreases the boiling point of the derivatives. The peaks are well separated and the last peak, $cys_2$, is eluted from the column after only 43 minutes. OV-1, a non-polar silicone, is thermally highly stable and its low bleeding let it appear suitable for GC-MS combinations.

Recently a single derivatization, single injection method for protein amino acids as their trimethylsilyl derivatives has been established (13). However, this method has not yet been developed to a level that permits carefree routine GC analysis.

In our studies we chose to derivatize the amino acids as their N-TFA isopropyl esters. The isopropyl esters

hydrochlorides are stable under anhydrous conditions. The free isopropyl esters are not as volatile as methyl esters, but they are volatile enough so that their retention temperatures are not too high. They can slightly more readily be prepared than n-butylesters. SF-96 was chosen as the stationary phase because it permits resolution of most of the variously structured amino acid derivatives (14). Due to its minor interference with the mass spectral analysis it is suitable for the use in GC-MS combination.

Resolution and separation of amino acid enantiomers by gas chromatography:

Amino acid enantiomers can be separated by GC by two different methods:

1. derivatization--esterification or acylation--of the enantiomeric amino acid with optically active reagents in order to form diastereoisomers and chromatography on an optically inactive phase
2. derivatization with an optically inactive reagent and chromatography on optically active stationary phases.

1. The amino acids can be converted into diastereoisomers by introducing a second optically active center e.g. by reaction of the carbonyl group with a secondary alcohol, a secondary amine or an α-amino acid. The second asymetric group may also be introduced by reaction of the amino group with e.g. N-TFA-prolyl chloride or an α-haloacid chloride.

For chromatography however the remaining functional group must also be derivatized. Various methods have been proposed. Many methods provided excellent resolvability of some enantiomers. However, the requirement of any procdure is its applicability to a complex misture of amino acids with various chemical structures. Thus far the widest range of amino acid diastereoisomers was resolved by Pollock and Oyama (15). They chromatographed the N-TFA-O-2-butyl esters on a 150' x 0.02" stainless steel capillary column coated with Carbowax 1540. Protein amino acids that were not resolved were try, arg, his, and $cys_2$. Furthermore aspartic acid merely shows a small resolution. A resolution of asp acid and try was later reported by Pollock (16) using a different derivative--either N-TFA 2, 2-dimethyl-3-butyl ester or N-pentafluorobenzoyl 2-butyl ester. The separation factor for some different amino acids is still low. The peaks for some derivatives overlap e.g. ser and pro and ile and aile.

If a third functional group is present in the amino acid further difficulties arise. The resolution factors for mono-TFA derivatives is higher than for di-TFA derivatives, which are normally formed in trifunctional amino acids. O- and S-TFA derivatives are not stable on certain stationary phases like Ucon LBX. A way to get around this is to form different derivatives of every functional group. This requires a multi step derivatization procedure.

Another way to introduce a second asymmetric center to form diastereoisomers is the reaction with N-TFA-prolyl chloride to form N-TFA-L-prolyl dipeptide methyl esters. If a third functional group is present, it again has to be converted into a different derivative.

Also in the latest available publication on amino acid diastereisomers, the resolution of the same seventeen amino acid pairs was achieved that had been resolved by Pollock. The amino acid enantiomers were resolved as their dimethyl-2-butyl-N-TFA derivatives on the relatively unpolar OV-17 packed columns. "Arg, his and $cys_2$ derivatives were not studied" (17) and remain still unresolved. The advantage of the reported method is that hydroxy and sulfhydryl amino acids need not to be derivatized by an additional second acylation step.

Possible racemization during the formation of N-TFA-amino acid esters and their gas chromatography was studied by Gil-Av (18). Esterification of D-ala, L-pro and L-met with optically pure D-octanol was carried out and no second chromatographic peak was detected.

Pollock et al (19) studied the effect of elevated temperature on racemization. They heated a leucine derivative up to 175°C for one hour. They did not observe any measurable racemization and concluded that diasteriomeric N-TFA amino acid esters are sufficiently thermally stable. In a very brief racemization study with N-TFA amino acid D-2-butyl

esters, we noticed partial racemization when a higher concentration of HCl is used for the esterification process (Fig. 1). However, indications of second peaks were also observed at low HCl concentrations. These peaks are either due to antipodic impurities of the reagent used in the derivatization procedure or to a small amount of racemization. The presence of these second peaks is at least advantageous in that respect that they represent an aid for peak identification. On the other hand in samples in which an enantiomeric compound is accompanied by a small amount of its antipode, the accurate determination of this antipode will be more difficult.

The method based on the separation of diastereoisomers requires a derivatizing agent of high optical purity, which cannot always be attained. If only small amounts of the antipode are present in the sample, a large error will be introduced in the calculation of the results.

2. The alternative approach, the separation of D and L amino acids by optically active stationary phases will probably in the future largely replace the separation diastereomeric derivatives. The best resolution for the D and L amino acids has been obtained by using synthetic dipeptides as solvent or stationary phase and the amino acids (solutes) as N-TFA alkyl esters (20, 21, 22 and references therein). Gil-Av and Feibush (23) were the first to report the separation of amino acid enantiomers on N-TFA-dipeptide ester phases.

FIGURE 1

N-TFA D-2-butyl esters of aliphatic DL- L-amino acids respectively (in the sequence of elution: α-aba, val, leu, nleu)

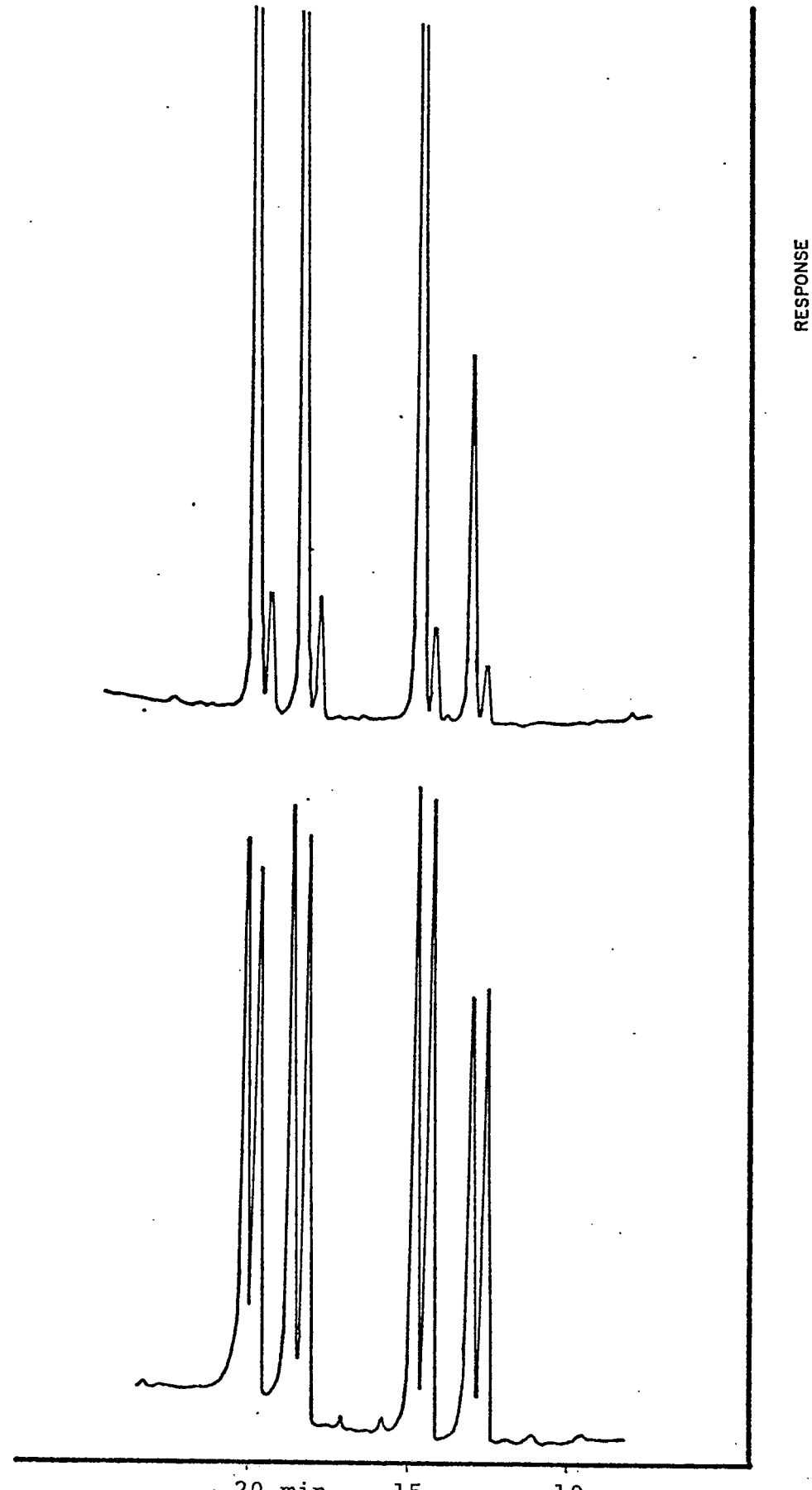
Derivatization: 5N HCl/D-2-Butanol at 100°C for 35 minutes

Column: 250' UCON 90,000

Isothermal at 140°C Carrier Gas: He 20 psi

Injection port 240°C

FID 290°


RESPONSE
TIME
20 min
15
10

Various other combinations of amino acids in dipeptide phases have been investigated since Gil-Av's initial investigations (23). The dipeptides are derivatized as N-TFA dipeptide cyclohexyl esters for the use as stationary phases since they have low melting points as well as low volatility. The separation of the derivatized amino acid enantiomers has been accounted for by the formation of a hydrogen-bonded diastereomeric association complex between solute and solvent. An increase in branching of the ester portion of the solute causes a better resolution of the D, L enantiomers. For instance the t-butyl esters of N-TFA-D, L-amino acids are better separated than the corresponding primary butyl esters. However, the secondary esters--usually isopropyl esters--were mostly used in practice because they are more readily synthezised, and they combine reasonable retention times with good resolution factors.

Because of limitations imposed by the maximum permissible working temperature of the phases, a mixture of protein amino acids is best analyzed in two steps. A 400 ft column coated with N-TFA-L-val-L-val cyclohexyl ester allows for the separation of 9 pairs of protein amino acids when both N-TFA and N-PFP-derivatives are used in separate injections. Less volatile amino acids have to be resolved on columns of 100 ft coated with N-TFA-L-phe-L-leu cyclohexyl ester. The only suitable derivatives for these four pairs of enantiomers are more volatile. The separation of $cys_2$ does not really

represent a problem. Cystine can readily be reduced to cysteine. The only amino acid that cannot be resolved up to now is his (21).

# II.

# EXPERIMENTAL

# II. EXPERIMENTAL

Equipment:

For the determination of the optimum derivatization conditions and retention times, a Varian $1200^{-1}$ gas chromatograph equipped with a FID detector was used. This instrument is designed for the use of packed columns. In order to decrease the diffusion in the injection port, the volume of the injection port was diminished by means of a glass liner. This was easily exchangeable and thus the injection system could be kept clean.

The mass spectral analyses were directly carried out in an LKB 9000 gas chromatograph-mass spectrometer combination.

The chromatographic column was a 300', 0.01" stainless steel needle stock. Short pieces of 0.03" tubing were welded to both ends of the capillary column and Swagelok fittings attached. New stainless steel tubing was rinsed with various solvents and dried by means of a flow of dry nitrogen. The column was then coated with a solution of 5% SF 96 and 0.25% Igepal in $CHCl_3$ at a pressure of 10 psi resulting a wating speed of $\sim$40 mm/sec. The column was dried from the solvent by continuing the flow of dry nitrogen and then slowly heated up to 200°C at 0.5°/min. After conditioning for 24 hours, the column was ready for chromatography.

The elution of arg, try, his and $cys_2$ was achieved and their decomposition during chromatography prevented by the

use of a unpolar shorter column. We used a 1.5 m x 0.2 cm glass column packed with OV101 on Chromosorb Q. This column could be operated at the same thermal conditions as the SF 96 column. This would allow simultaneous operation of both columns in a dual column chromatograph. In order to avoid partial decomposition, especially of polyacyl derivatives in the injection port, the coinjection of trifluoroacetic acid anhydride (TFAA) was necessary. Due to the low capacity of the 0.01 inch capillary column it was not possible to inject the dilute samples directly. Therefore, the cryogenic injection technique was applied. The sample was trapped in the first few turns of the column which was kept at room temperature. After due time for trapping the sample (4 min) the column was rapidly heated to the initial temperature of the temperature program and the program started.

Reagents:

The amino acids were obtained from Nutritional Biochemicals, Schwarz Laboratories, Mann Research Laboratories, and K and K Laboratories Inc., and were chromatographically pure. A solution containing 1 mg/ml of each amino acid in 0.1 N HCl was used as a stock solution for preparing the derivatives.

Hydrocarbon reference standards from Theta Corporation were used for the determination of the retention time in methylene units.

The trifluoroacetic anhydride was obtained from Eastman Kodac Co. and was redistilled over $P_2O_5$. Isopropanol and chloroform were dried over molecular sieve and were redistilled from an all-glass system. The hydrogen chloride gas CP grade was filled by Union Carbide Corporation. The HCl gas was passed through two sulfuric acid drying towers before bubbling into the ice-cooled isopropanol. The normality of the HCl in isopropanol was determined by weight and confirmed by titration.

Derivatization method:

The complete derivatization procedure of an amino acid was conducted in one and the same glass reaction vial to prevent contamination and contact with the atmospheric moisture. Teflon-lined screw caps were used to tightly close the vials during the estrification and acylation steps. Vacuum was used for evaporation of solvents and excess reagents from the samples. For this purpose a glass tube was introduced into the top of the vial and the joint tightly sealed by means of "Strip Teeze" teflon tape.

A 110° constant-temperature sand-bath was used to heat the samples during esterification and acylation of the amino acids. The solvents of the esterification step were evaporated in a 50° sand-bath.

Hamilton syringes (100 and 10 µl) or glass micropipets were used for the addition of the derivatization reagents

to the samples. A 1µl microsyringe served for the injection of the samples onto the gas chromatograph.

The following procedure was used for the derivatization of amino acids as their N-TFA-isopropyl esters.

1. An aliquot of an acidic solution (0.01N HCl) containing one or a mixture of amino acids was placed in a vial and evaporated just to dryness in a 110°C sand bath under a stream of nitrogen gas. After all visible moisture had been removed from the vials, 100 µl of anhydrous chloroform were added and evaporated under vacuum to azeotropically remove the last traces of moisture.

2. For esterification of the amino acids 0.6 ml of isopropanol 3N HCl were added to the reaction vials. The vials were then tightly capped and placed in a 110° sand bath for 1 hour. Only the lower portion of the vial was submerged in the sand, allowing the sample to reflux. The sample was then allowed to cool, placed in the 50° sand bath, and evaporated just to dryness under vacuum. 100 µl of chloroform were added and evaporated to azeotropically remove traces of reagents.

3. The amino acid isopropyl esters were trifluoroacetylated by addition of 0.2 ml chloroform and 0.4 ml of TFAA. The vials were again tightly capped and refluxed in the 110° sand bath for 10 minutes.

After allowing the sample to cool the solvent and excess reagents were carefully removed at room temperature

and reduced pressure. The N-TFA-ester of the amino acid thus prepared was taken up to 50 µl of chloroform and 0.1 µl portions was used for GC and GC-MS.

Ion exchange:

The amino acids formed in the hydrogencyanide polymerization experiment by J. P. Ferris were isolated from the fractionated polymerizates by ion exchange. Triple distilled water was used throughout the procedure. Each sample of the dried hydrolysate was dissolved in about 3 ml of water and charged on a column with 12 ml Dowex 50 (Bio Rad) ion exchange resin, prepared in the $H^+$ form. Dowex 50 ($H^+$) allows for the separation of amino acids and bases from organic acids and nonionics. The column was successively eluted with six bed volumes of water and six bed volumes of 2N $NH_4OH$. The $NH_4OH$ eluate was collected, evaporated to dryness and redissolved in 5 ml of water. This solution was charged on a column with 12 ml Dowex 1 ($OH^-$) ion exchange resin. Dowex 1 allows for the separation of amino acids from bases. The column was successively eluted with six bed volumes of water and six bed volumes of 1N HCl. The eluate was collected, evaporated to dryness and finally kept under high vacuum (0.5 mm Hg) for 20 minutes. Derivatization of the samples was carried out as described above.

# III.

# RESULTS AND DISCUSSION

# III. RESULTS AND DISCUSSIONS

Derivatization procedure:

For satisfactory analysis by gas chromatography, an essentially complete derivatization is necessary.

The optimum esterification time was established for three types of amino acids, aliphatic, hydroxy, and sulfur containing amino acid. Ile, ser and met were reacted with isopropanol/HCl for various durations of time at 110°. The time of trifluoroacetylation was kept 5 minutes at 110° consistently for each sample. The optimum gas chromatographic peak was obtained after 45 minutes of esterification of a 50 μg sample for either of the three types of amino acid. In addition, the effect of prolonged evaporation after removal of solvents of the esterification reaction was studied. Additional heating for up to 20 minutes did not produce loss of either type of the three esterified amino acids due to the nonvolatility of the amino acid ester hydrochlorides.

The loss of N-TFA isobutyl derivatives of amino acids by Gehrke (28). This data shows that losses occur when evaporated at 100°, however relatively low losses were observed when dried at 25°. To carry this further we studied the effect of prolonged evaporation at 100° for two 50 μg samples of derivatized aliphatic and hydroxy amino acid. An appreciable loss of the aliphatic amino acid derivative was already observed if the sample was kept at 100° just

3 minutes after attaining the dry derivative. After prolonged evaporation for 20 minutes the 50 µg of aliphatic amino acid had almost vanished. The sensitive hydroxy amino acid derivative had been decomposed upon heating just 3 minutes after removal of solvents.

Further losses of amino acid derivatives occur due to their sensitivity to moisture. Especially the N-TFA derivatives of hydroxy and mercapto amino acids were found not to be stable to moisture. The breakdown of cys, hyp, ser, thr and tyr occurs relatively rapidly.

The thermal stability of derivatives under the conditions of gas chromatography has not been quantitatively investigated. One can merely expect that no appreciable decomposition during gas chromatography occurs as long as narrow nontailing peak are obtained. The N-TFA isopropyl esters of $cys_2$, try, his, and arg were chromatographed on a glass column packed with an unpolar phase (OV 101), in order to prevent the decomposition of these extremely sensitive derivatives. The coinjection of trifluoroacetic acid anhydride proved to be necessary to facilitate recombination.

The amino acid isopropyl esters were sufficiently soluble in TFAA. The try ester underwent side reactions and turned to various colors when dissolved in conc. TFAA. A better way to react the try ester was to dissolve the ester in TFAA-chloroform (1:9).

FIGURE 2

Optimum esterification time

50 µg Ile esterified for 3, 6, 12, 20, 30, 45 and 120 min. at 110°.

Acylation 5 min. at 110° for each sample.

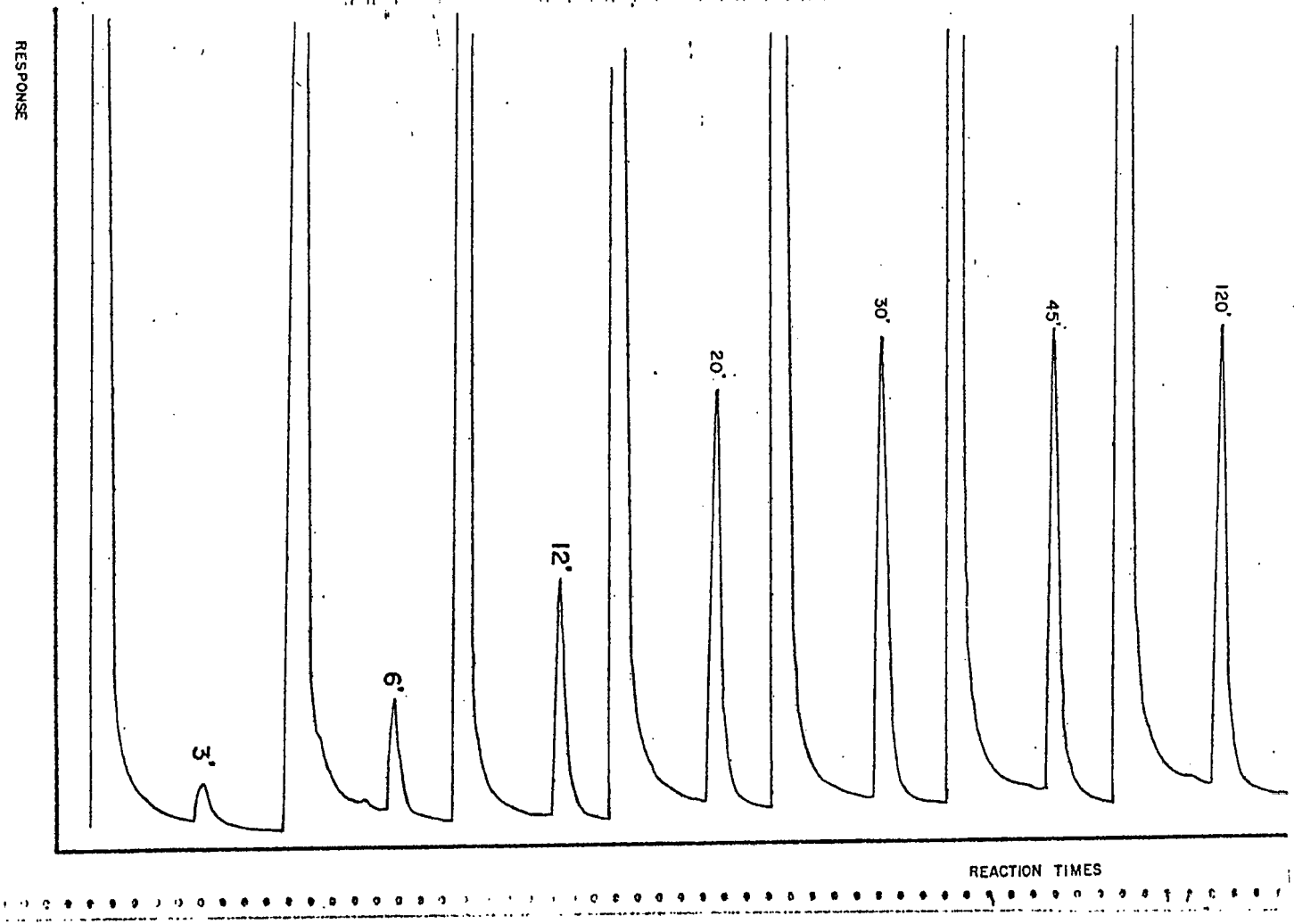
GC 140° isothermal

Injected 5 µg/5 µl

Injection port 240°C

Detector 290°C

Carrier gas He 4cc/min


RESPONSE
3'
6'
12'
20'
30'
45'
120'
REACTION TIMES

Arg isopropyl ester seemed to decompose if trifluoroacetylated at 120°. Its TFA derivative was thus prepared at room temperature. It was found to be necessary especially for hydroxy and sulfhydryl groups containing amino acids to exclude traces of isopropanol--by azeotropic evaporation with chloroform--prior to the fluoroacetylation step in order to obtain reproducible results and to derivatize all groups in the molecule.

Retention time in methylene units:

The retention time of N-TFA amino acid isopropyl esters in methylene units was determined by coinjection of a small number of N-TFA amino acid isopropyl esters and a standard of normal alkanes. One methylene unit is the distance between two consecutive n-alkane peaks. The retention time of a N-TFA amino acid ester was related to the preceeding hydrocarbon peak and expressed as the carbon number of this hydrocarbon plus the distance to the chromatographic peak in extrapolated decimals of one methylene unit.

It should be noted that the methylene unit for amino acid derivatives presented in Table 1 and given in one tenth of one methylene unit can only be reproduced under exactly the same gas chromatographic conditions. Any change of parameter like temperature program speed, column material, relative concentration of alkanes and derivatized amino acid, will alter the retention time in methylene units.

However, even for non-standardized conditions, the methylene units will be an aid to "locate" an amino acid in the chromatogram and to identify it.

Mass spectrometry:

The mass spectra of the ethyl esters of amino acids have been studied (29) and these investigations provided the first detailed information of the behavior of amino acid derivatives upon electron impact.

The mass spectra of 39 protein and nonprotein N-TFA isopropyl esters are presented in Figures 5-18. The mass spectrographic data of the protein amino acids and some of the nonprotein amino acids as their N-TFA n-butyl esters have already been reported. We therefore refer to references (30) and (31) for their discussion. The different alkyl group that was used in the esterification produces little changes in the fragmentation pattern of these amino acids.

The nonprotein amino acids already reported are α-, β-, γ- and N-methyl isomers of $C_3$ and $C_4$ aliphatic amino acids. It was shown by which criteria these types of amino acids can be distinguished. The α-amino acids are characterized by simple spectra with the loss of the alkylcarbonyl group accounting for the major peak. The derivatives of the β-amino acids have a more complex spectra in which not a single ion dominates the spectra as is found in the case of the α-amino acids. The γ-amino acids derivatives do show

FIGURE 3

Determination of the retention time in methylene units for four N-TFA amino acid isopropyl esters.

Injected: hydrocarbon standard nonane to hexadecane; 10 μg of each amino acid.

Column: SF 96 300 ft x 0.02 in. I.D. glass

Conditions: cryogenic injection, 5 min. initial hold, program rate 1°/min. 80 200°C, range 4 x $10^{-10}$

Carrier gas He 20 psi

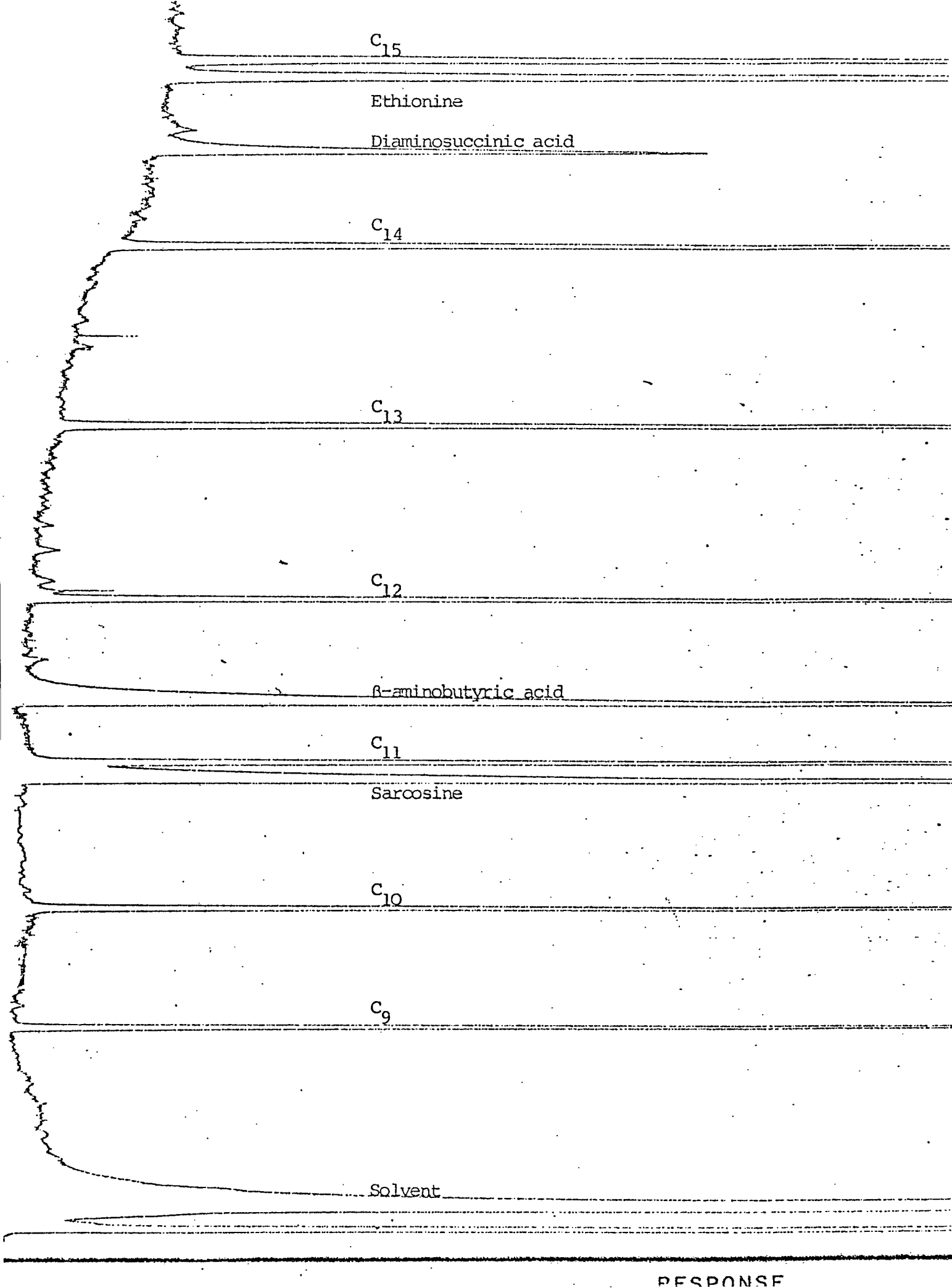

$C_{15}$
Ethionine
Diaminosuccinic acid
$C_{14}$
$C_{13}$
$C_{12}$
ß-aminobutyric acid
$C_{11}$
Sarcosine
$C_{10}$
$C_9$
Solvent
RESPONSE

TABLE 1

Retention Time in Methylene Units for N-TFA Isopropyl Esters of Protein Amino Acids on SF 96 Stainless Steel Needle Stock 300', 0.01".

| | |
|---|---|
| Alanine | 10.4 |
| Glycine | 10.9 |
| Threonine | 11.3 |
| Valine | 11.4 |
| Serine | 11.5 |
| Leucine | 12.1 |
| Isoleucine | 12.2 |
| Cysteine | 13.0 |
| Proline | 13.3 |
| Hydroxyproline | 13.8 |
| Aspartic acid | 14.2 |
| Methionine | 14.7 |
| Glutamic acid | 15.4 |
| Phenylalanine | 15.6 |
| Lysine | 18.3 |
| Tyrosine | 18.9 |

TABLE 2

Retention Time in Methylene Units for N-TFA Isopropyl Esters of Nonprotein Amino Acids on SF 96 Stainless Steel Needle Stock 300', 0.01".

| | |
|---|---|
| α -Aminoisobutyric acid | 10.4 |
| α-Aminobutyric acid | 11.1 |
| Sarcosine | 11.2 |
| Isovaline | 11.3 |
| β-Alanine | 11.8 |
| Norvaline | 11.8 |
| β-Aminobutyric acid | 11.8 |
| Isoserine | 11.9 |
| Allothreonine | 12.1 |
| Alloisoleucine | 12.2 |
| γ-Aminobutyric acid | 12.6 |
| β-Aminoisobutyric acid | 12.7 |
| N-Methylvaline | 13.0 |
| N-Methyl-γ-alanine | 13.2 |
| Norleucine | 13.2 |
| Diaminopropionic acid | 13.4 |
| Pipecolic acid | 13.6 |
| N-Methyl-β-aminobutyric acid | 13.8 |
| N-Methylleucine | 13.9 |
| Diaminosuccinic acid | 14.9 |
| Ethionine | 15.3 |
| 2,4-Diaminobutyric acid | 15.9 |

FIGURES 4-16

Mass spectra of N-TFA isopropyl esters of protein amino acids and amino acids found in the Murchison meteorite and S. L. Miller's recent electric discharge synthesis.

Instrument LKB 9000

Column SF 96 Length 300' I.D. 0.01"

Column temperature program rate 3°/min 80-200°C

He flow 4 ml/min at 40 psi

TIC monitor position 5 Chart paper 5 mm/sec

Flash heater temp. 215°C Separator temp. 225°C

Ionization current 60 μA

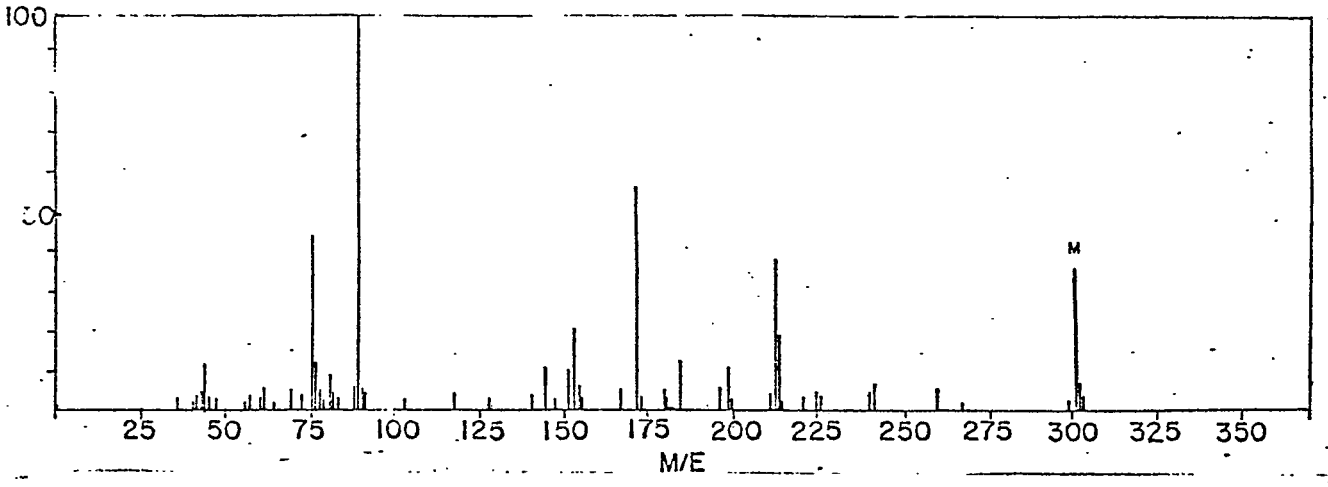
Acceleration volt. 3.5 kV

Multiplier volt. 2.9-3.3 kV

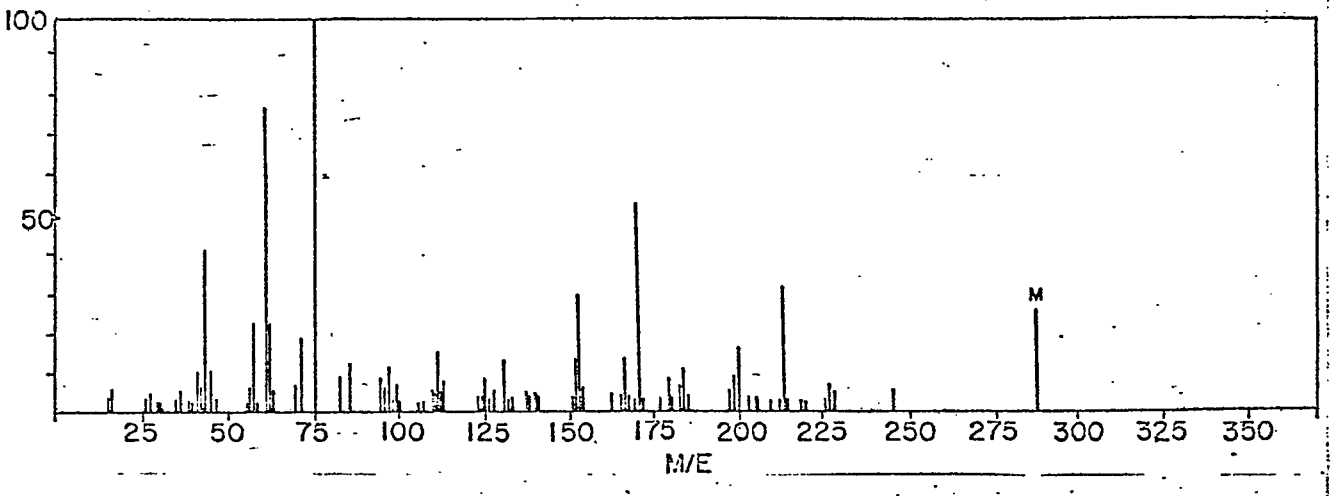
Analyzer pressure 2 x $10^{-7}$ mm Hg

Scan speed 5 UV-paper speed 4 inches/sec. Filter 120 cps

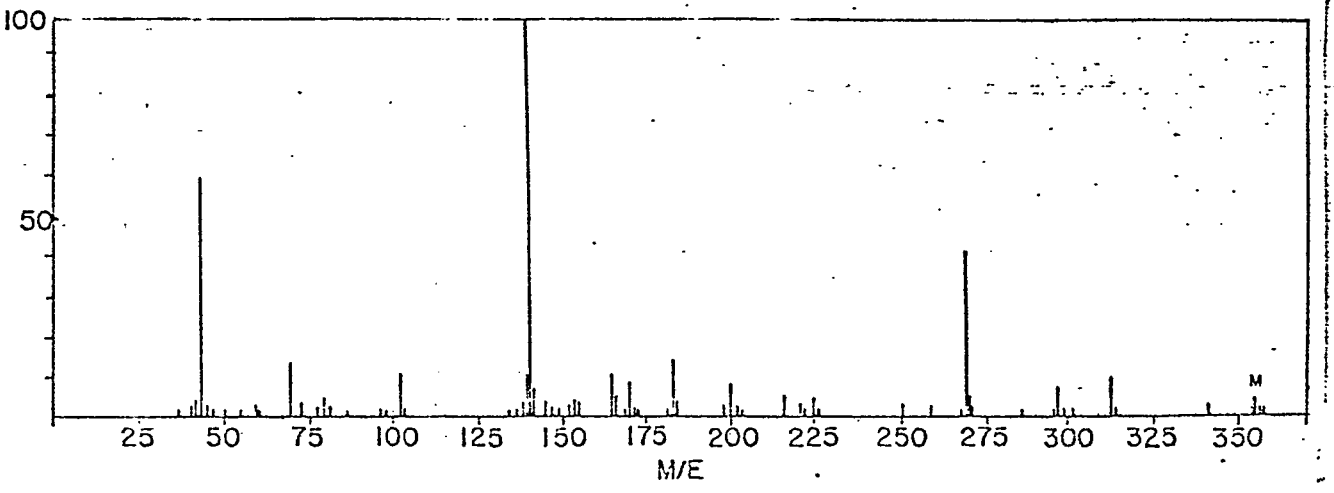
ETHIONINE




METHIONINE

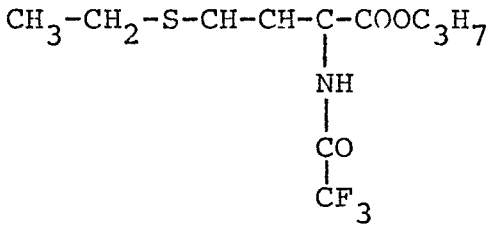



CYSTEINE



the loss of the alkylcarbonyl group as for α- and β- amino acids. The N-methyl amino acids have an ion at m/e 110 formed from the N-methyl TFA group which is characteristic to them.

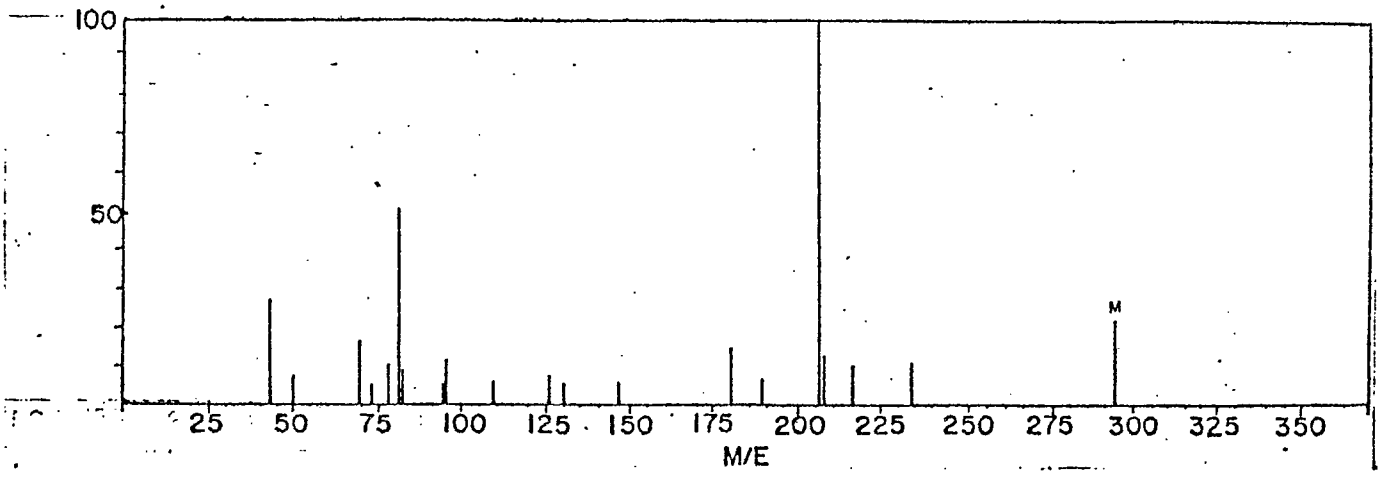
Mass spectral fragmentation of N-TFA-isopropyl ester of ethionine



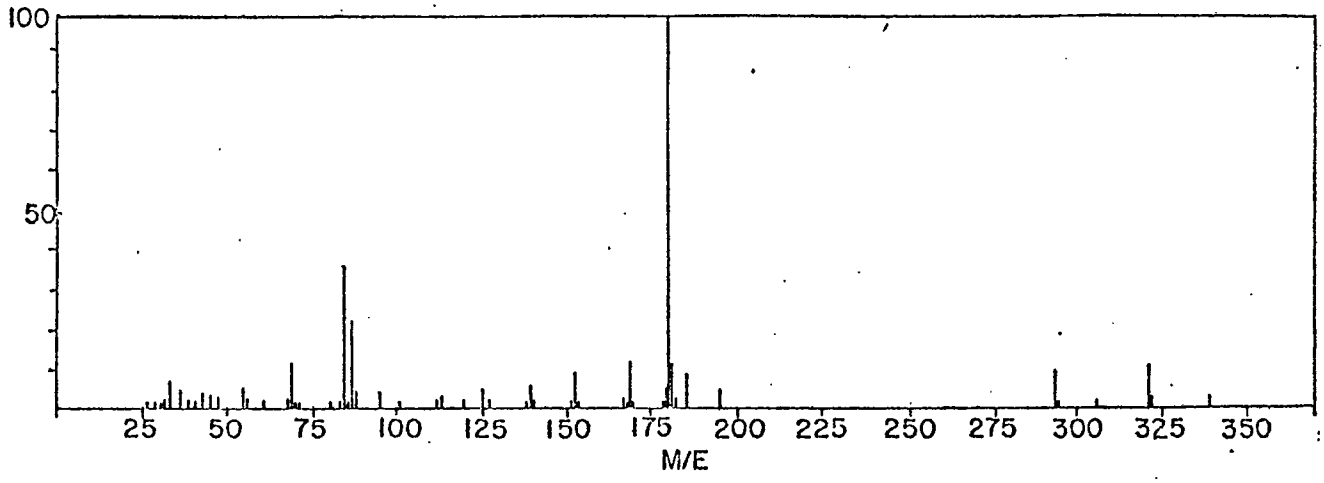
| | m/e | % |
|---|---|---|
| M | 341 | 36.0 |
| M-$CH_3$-$CH_2$-S-CH | 226 | 4.0 |
| M-$CH_3$-$CH_2$-S-CH=$CH_2$ | 213 | 40.0 |
| M-$C_3H_7COO$ | 214 | 19.0 |
| M-($C_2H_5S$ + $C_3H_6$) | 198 | 10.8 |
| M-($C_2H_5$-S-$CH_2$ + $C_3H_6$) | 184 | 11.2 |
| M-($C_2H_5S$ + $C_3H_7OH$) | 166 | 6.3 |
| M-($C_2H_5S$ + $C_3H_7COO$) | 153 | 22.0 |
| M-($C_2H_5SH$ + $C_3H_7COO$) | 152 | 9.4 |
| $C_2H_5S\cdot CH_2CH_4$ | 89 | 100.0 |
| $C_2H_5S\cdot CH_2$ | 75 | 47.0 |
| $CH_3S\cdot CH_2$ | 61 | 6.5 |

One immediately notices in this spectrum the unusually high intensity of the molecular ion peak (36%). In general

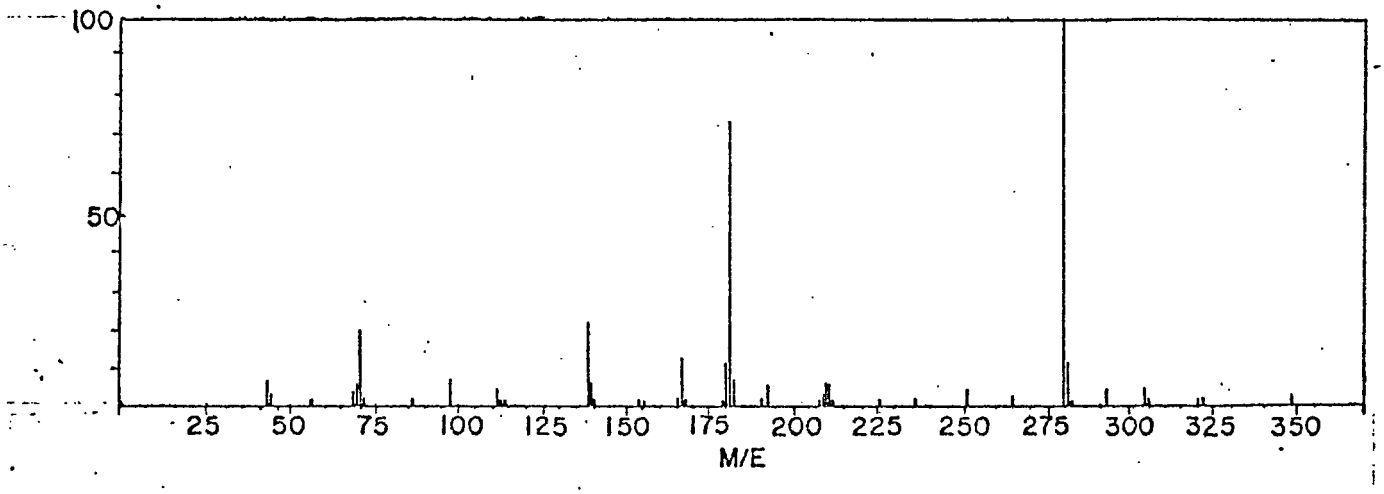
HISTIDINE



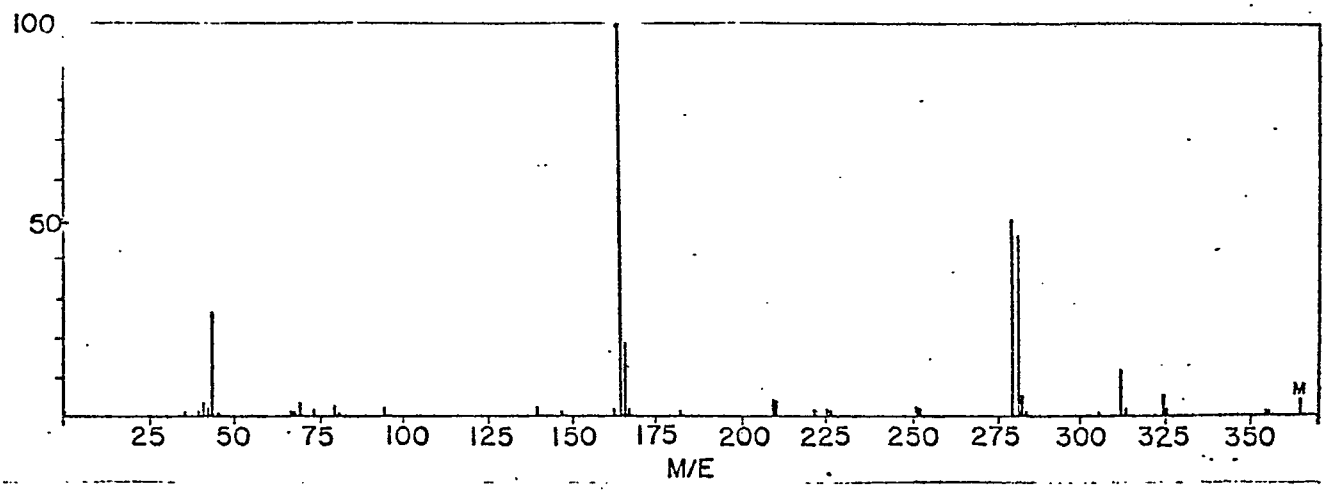

LYSINE



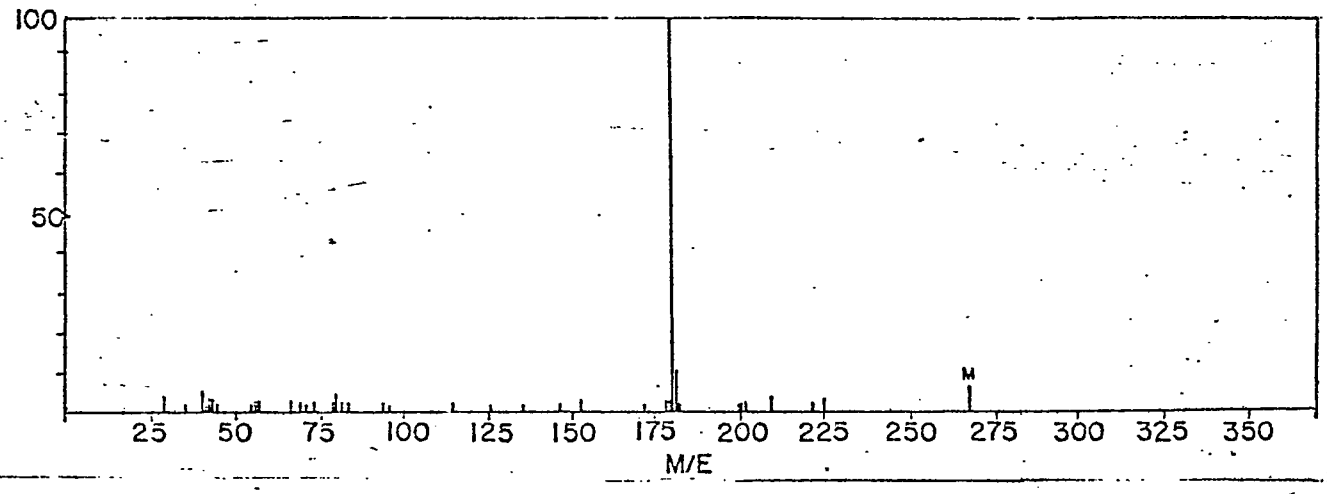

ARGININE



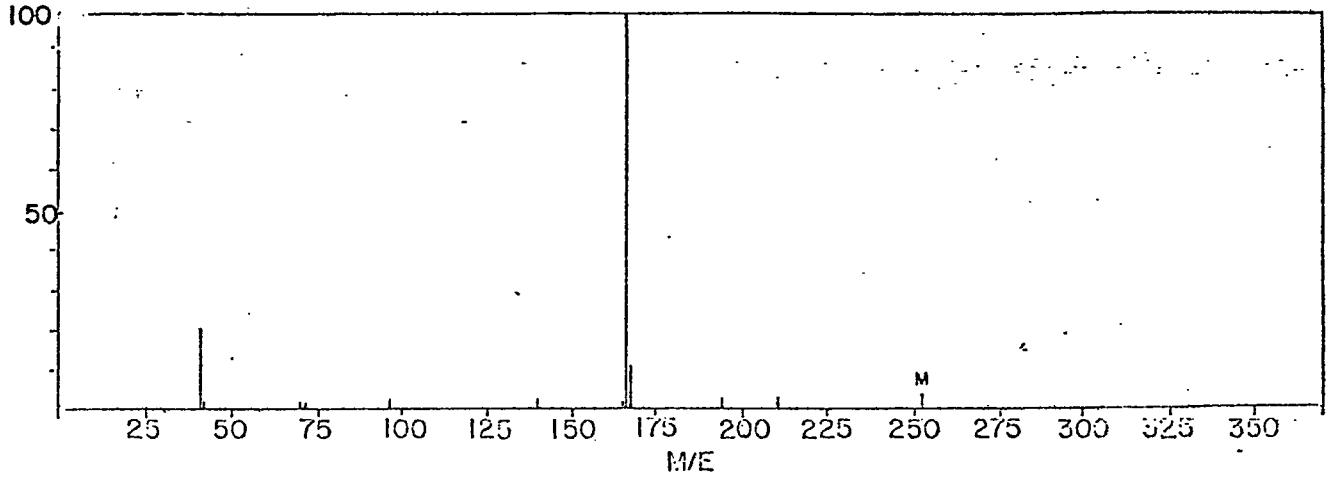
HYDROXYPROLINE




PIPECOLIC ACID

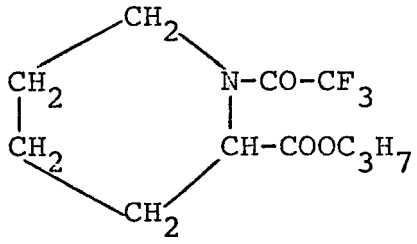



PROLINE



the most intense ions are derived from cleavage of bonds α and β to the sulfur. There again the formation of the iminium fragment by loss of $C_3H_7COO$ results in a fairly intense ion at m/e 214. Elimination of $C_2H_5S$ and $C_2H_5SH$ from the iminium ion results in fragments at m/e 153 and 152, respectively.

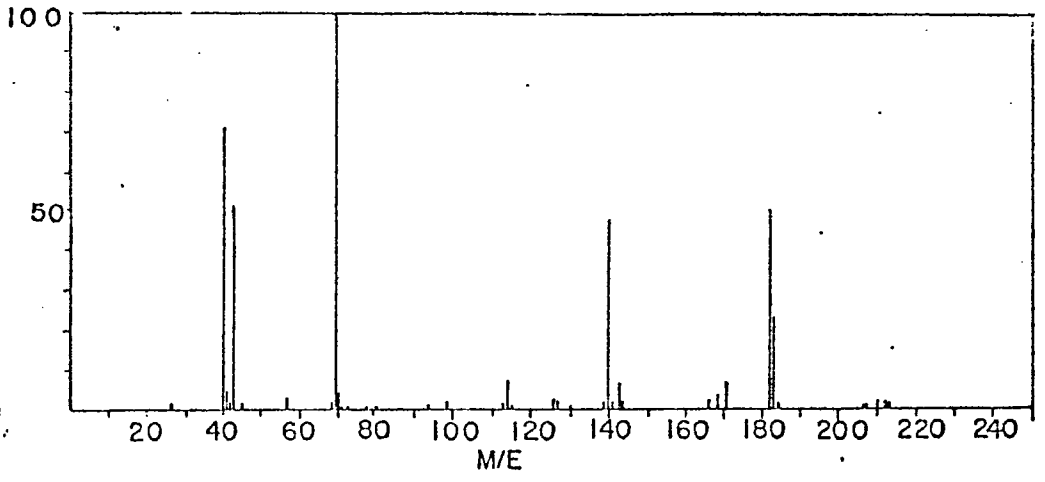
Mass spectral fragmentation of N-TFA-isopropyl ester of pipecolic acid



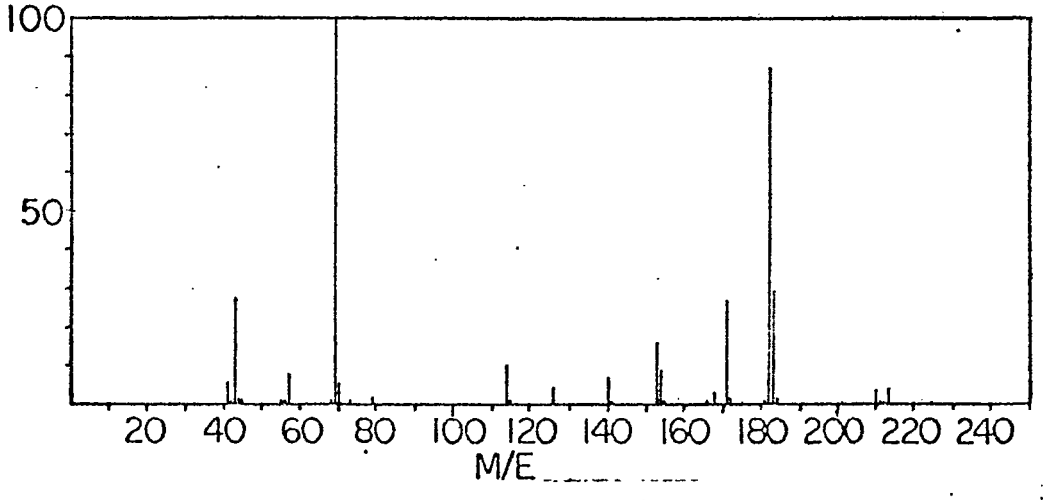

| | m/e | % |
|---|---|---|
| M | 267 | 6.8 |
| $M-C_3H_7O$ | 208 | 4.3 |
| $M-C_3H_6COO$ | 181 | 10.0 |
| $M-C_3H_7COO$ | 180 | 100.0 |
| $CF_3CO-N-CH_2-CH_2-CH_2$ | 153 | 2.2 |
| $CF_3CO-NH-CH_2$ | 126 | 1.9 |
| $CH_2-CH_2-CH_2-CH$ | 55 | 2.2 |

This spectrum is characterized by the small number and low intensity of its fragment ions which is due to the stability of the cyclic iminium ion (m/e 180). Another diagnostic peak occurs at m/3 153 and is formed from the iminium ion by the expulsion of ethylene.

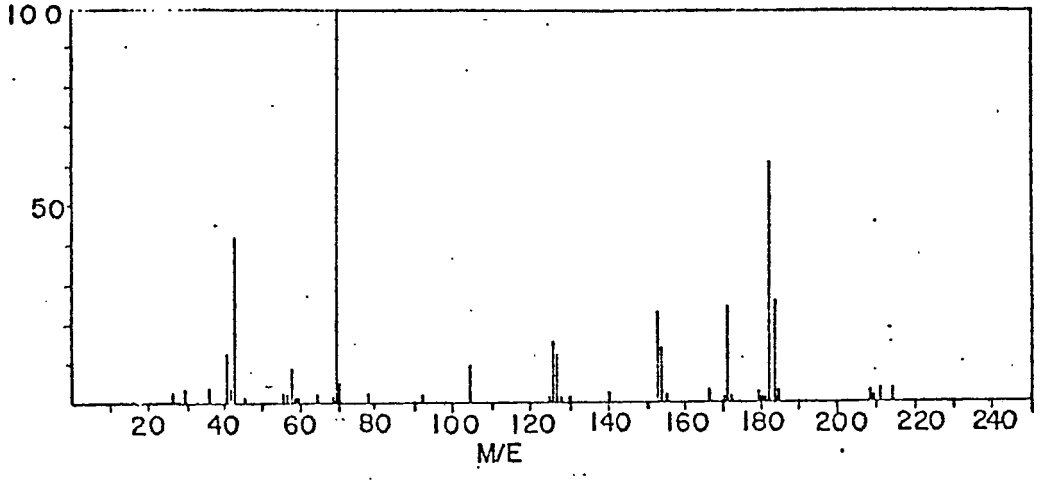
LEUCINE



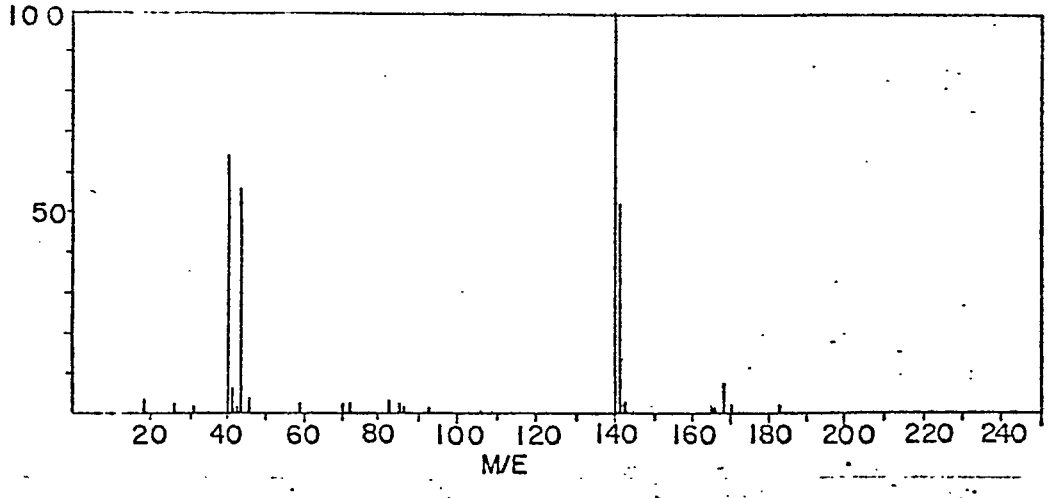

ALLOISOLEUCINE



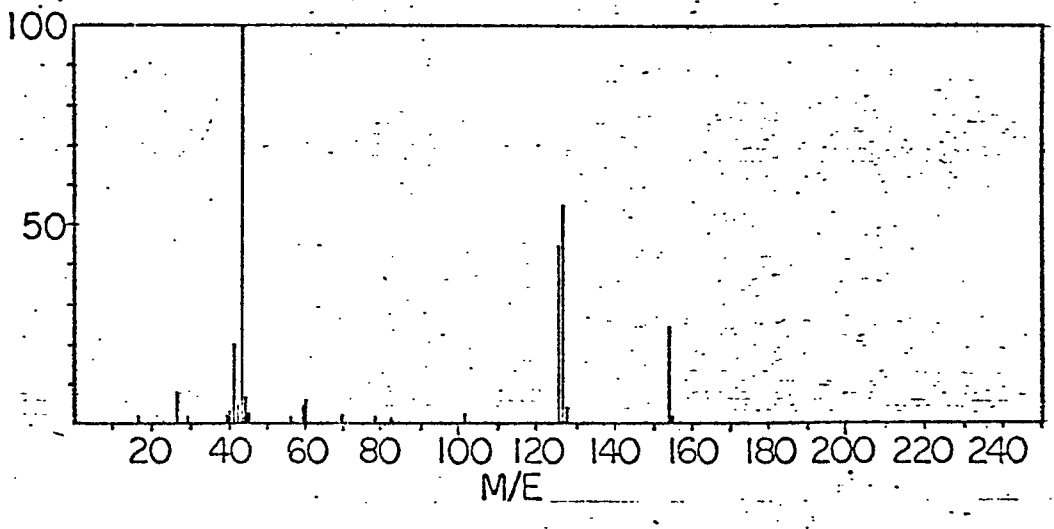

ISOLEUCINE



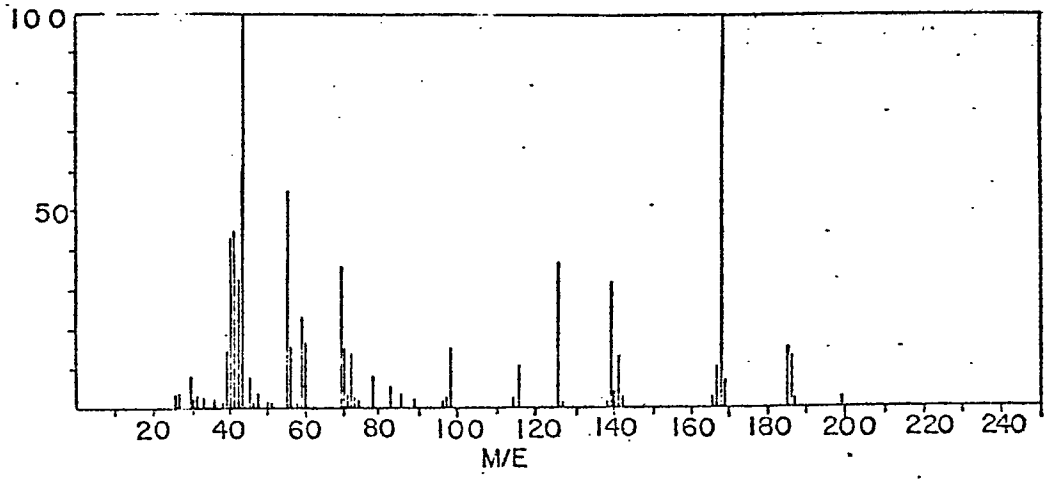
ALANINE



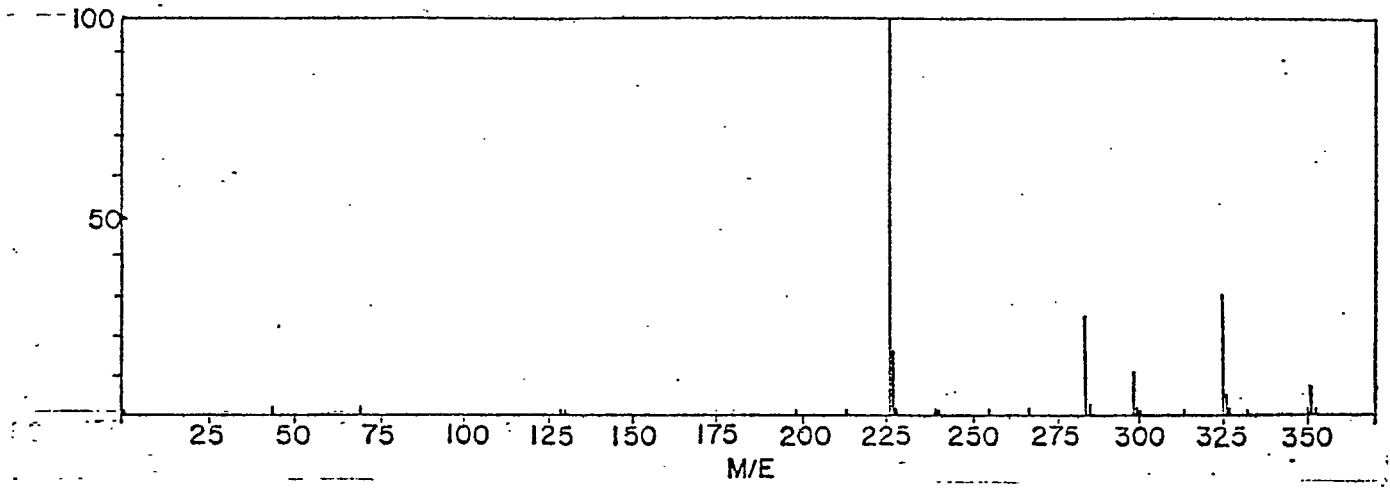

GLYCINE



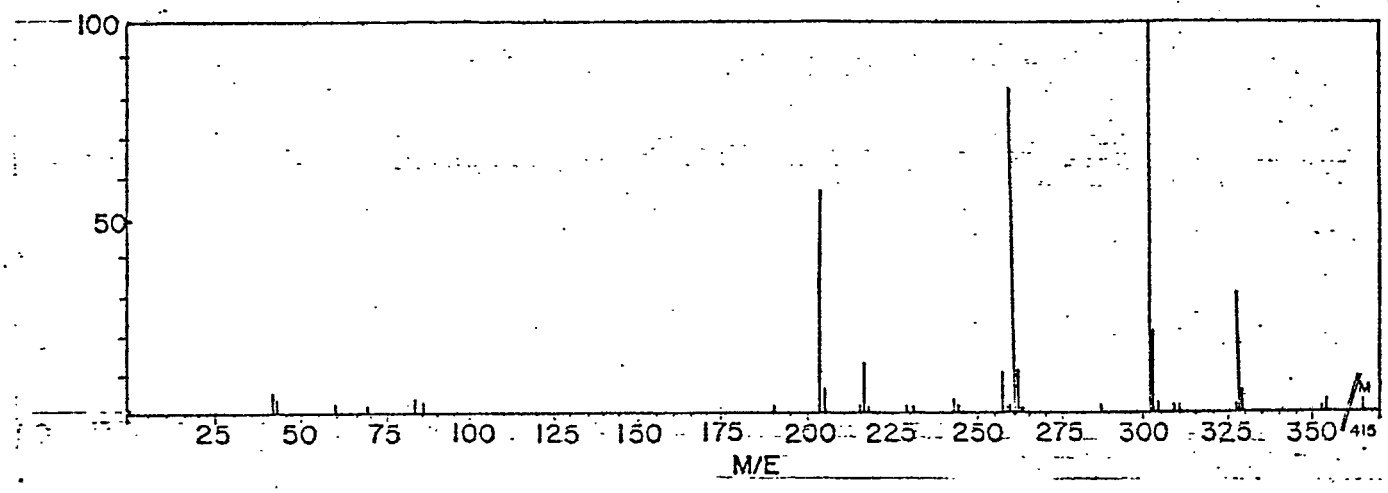

β -ALANINE



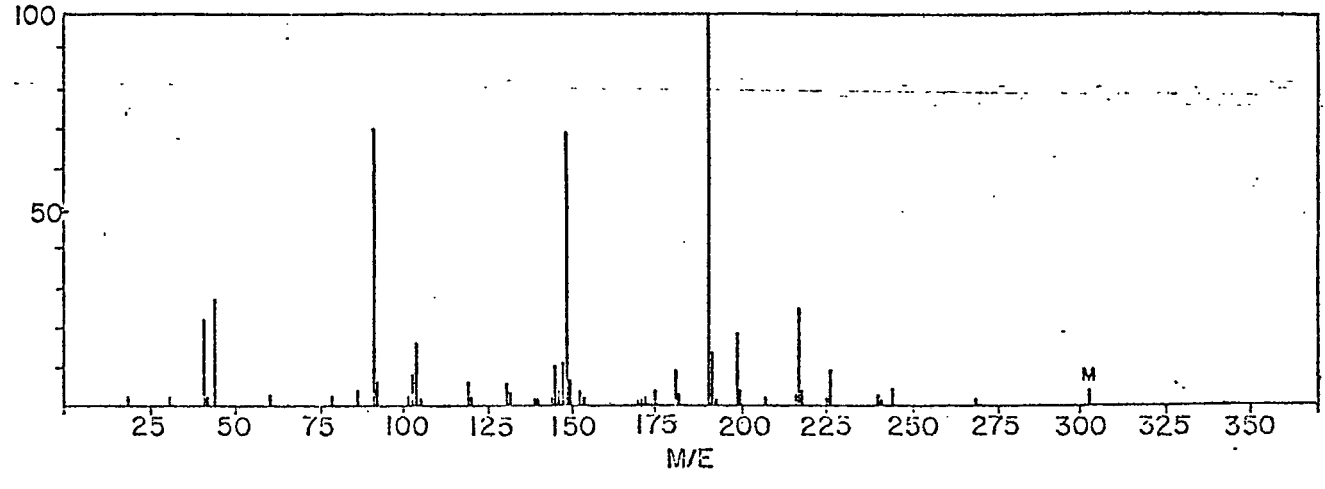
TRYPTOPHAN



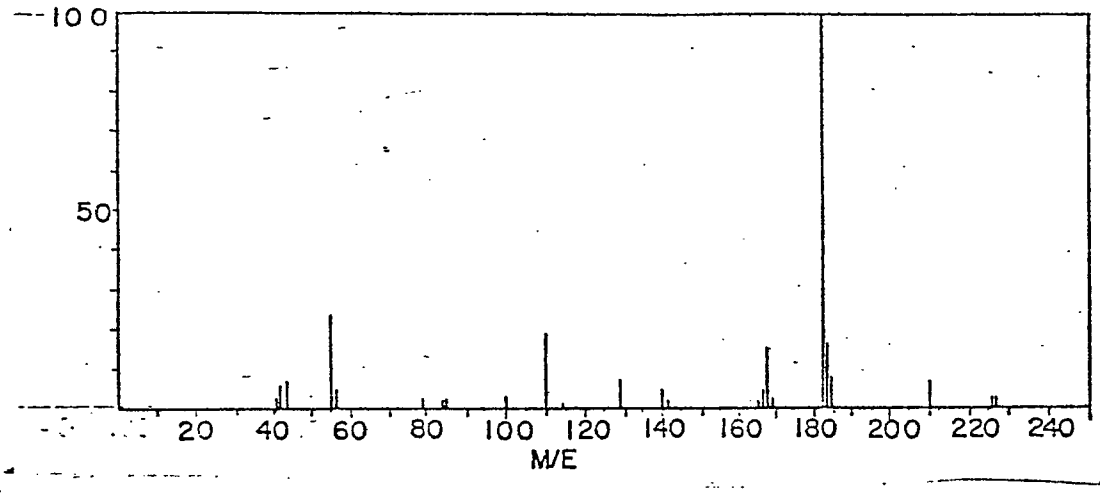

TYROSINE



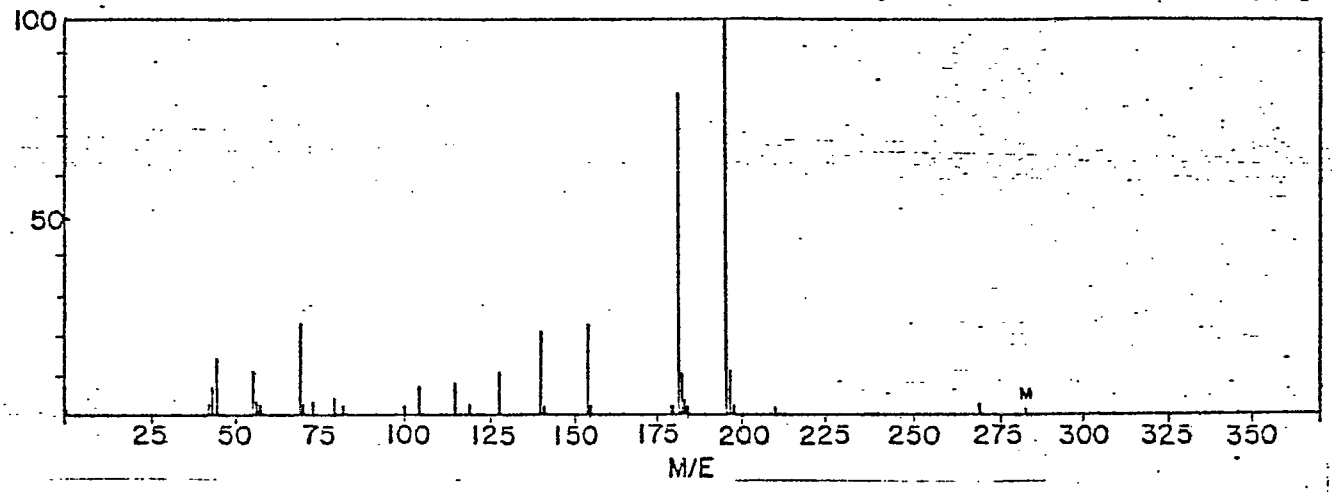

PHENYLALANINE



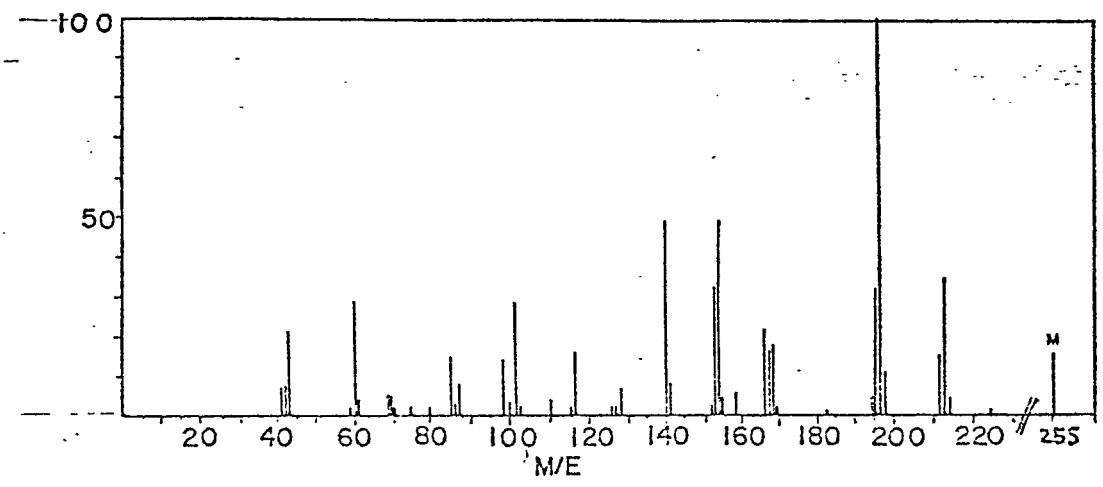
N-METHYLVALINE



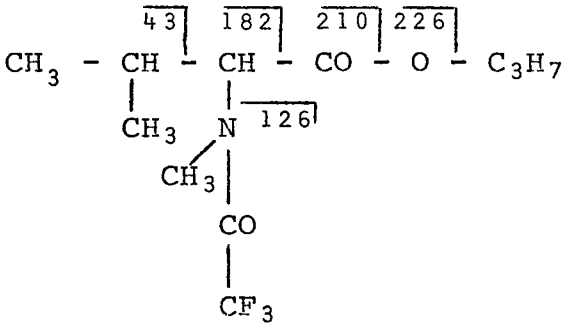

N-METHYLLEUCINE




N-METHYL-γ-AMINOBUTYRIC ACID

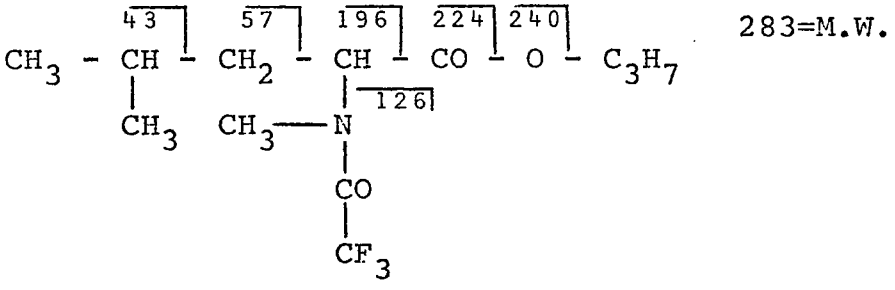


Mass spectral fragmentation of N-TFA-N-Methylvaline isopropyl ester



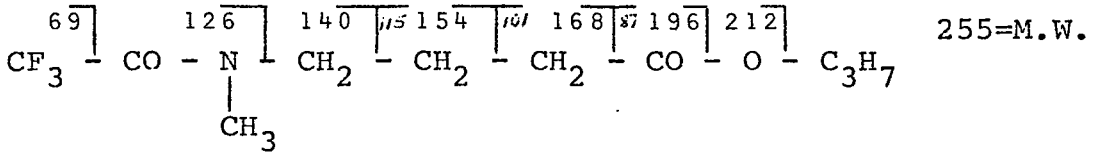

| additional fragments | m/e |
|---|---|
| 182 - $CH_2$ | 168 |
| $CF_3$ - CO - $N(CH_3)$ - $CH_2$ | 140 |
| $CF_3$ - CO - $NH_2(CH_3)$ | 128 |
| $CF_3$ - C = N - $CH_3$ | 110 |

Mass spectral fragmentation of N-TFA-N-Methylleucine isopropyl ester

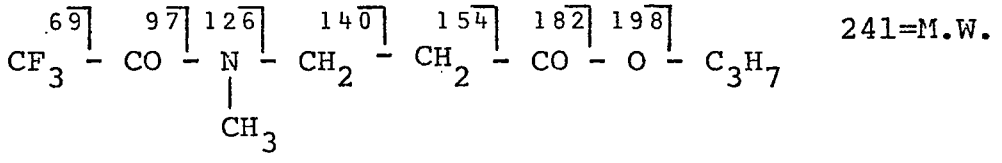



| additional fragments | m/e |
|---|---|
| 196 - $CH_2$ | 182 |
| $CF_3$ - CO - $N(CH_3)$ - $CH_2$ - $CH_2$ | 154 |
| $CF_3$ - CO - $N(CH_3)$ - $CH_2$ | 140 |
| $CF_3$ - CO - $NH_2CH_3$ | 128 |
| $CF_3$ - C = N - $CH_3$ | 110 |

Mass spectral fragmentation of N-TFA-N-Methyl-γ-aminobutyric acid isopropyl ester

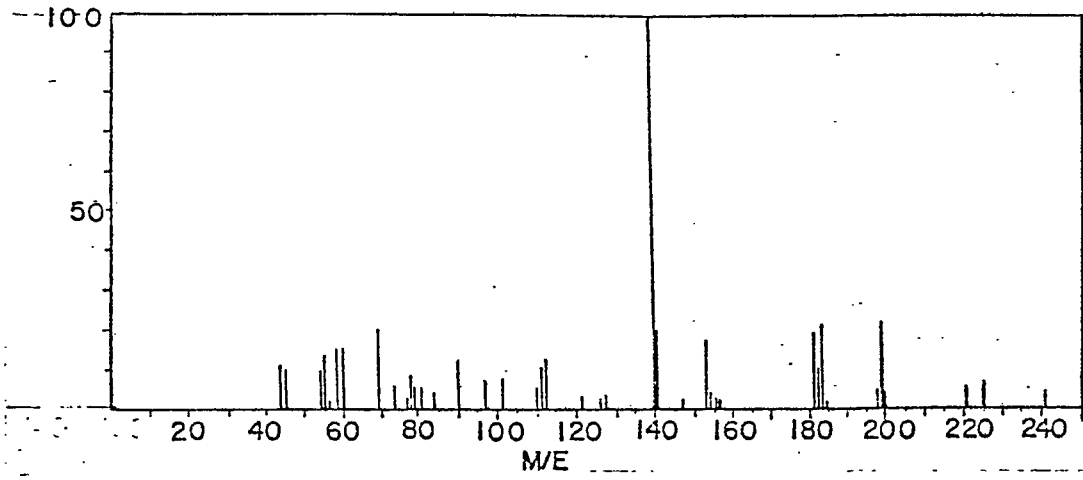



Mass spectral fragmentation of N-TFA-N-Methyl-β-alanine isopropyl ester

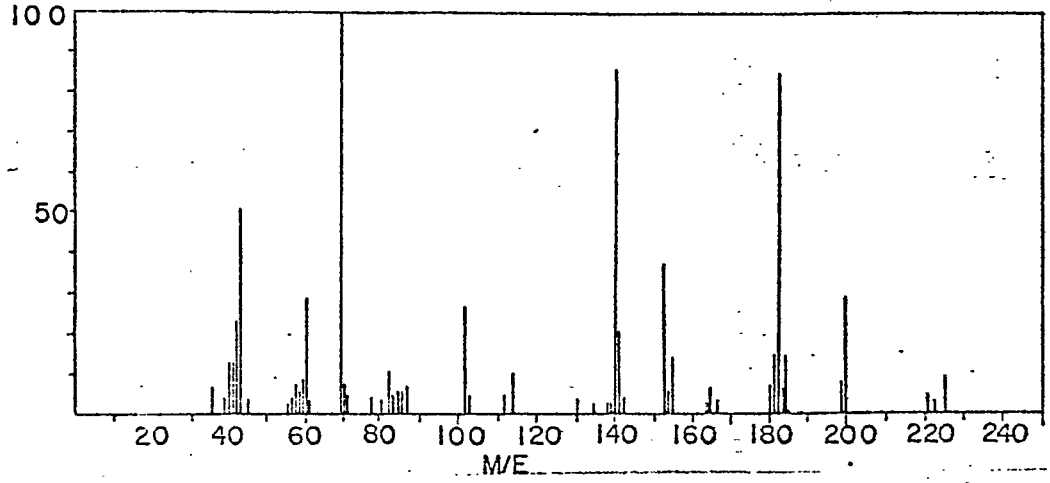



All the N-TFA-N-methyl amino acid isopropyl esters show an ion at m/e 110 $CH_3-\overset{(+)}{N}=C-CF_3$. Its presence cannot be observed in non-N-methyl amino acids.

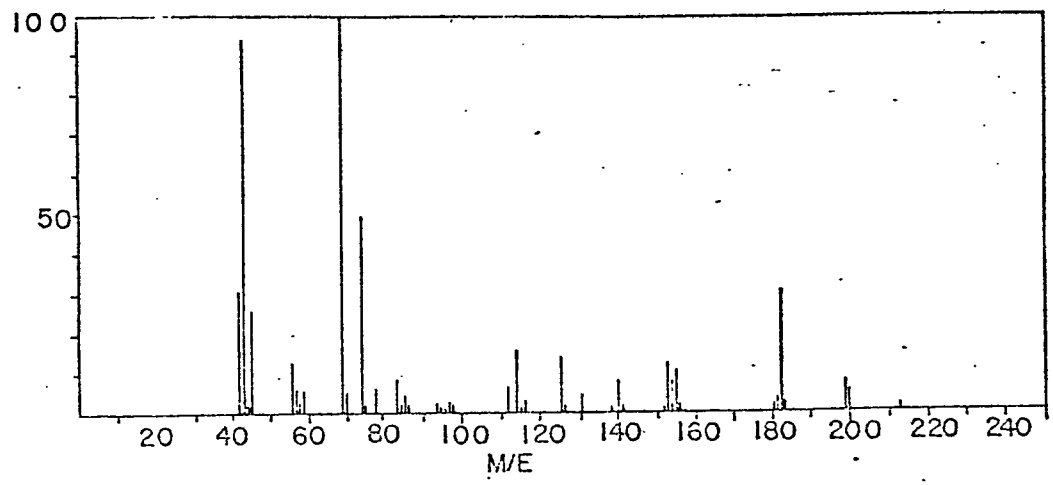
N-METHYL-β-ALANINE



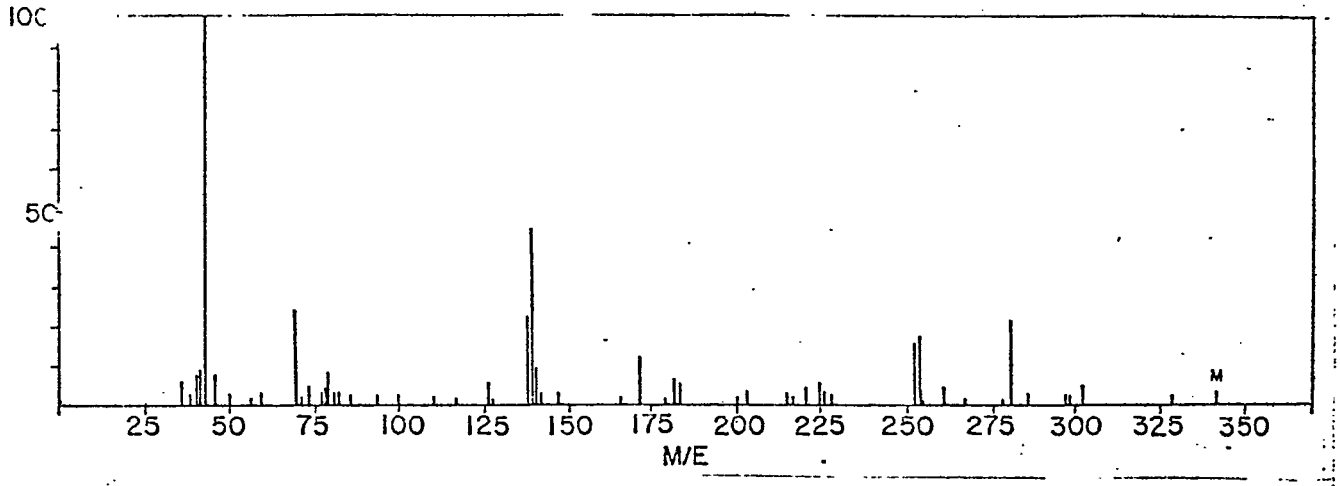

β—AMINOBUTYRIC



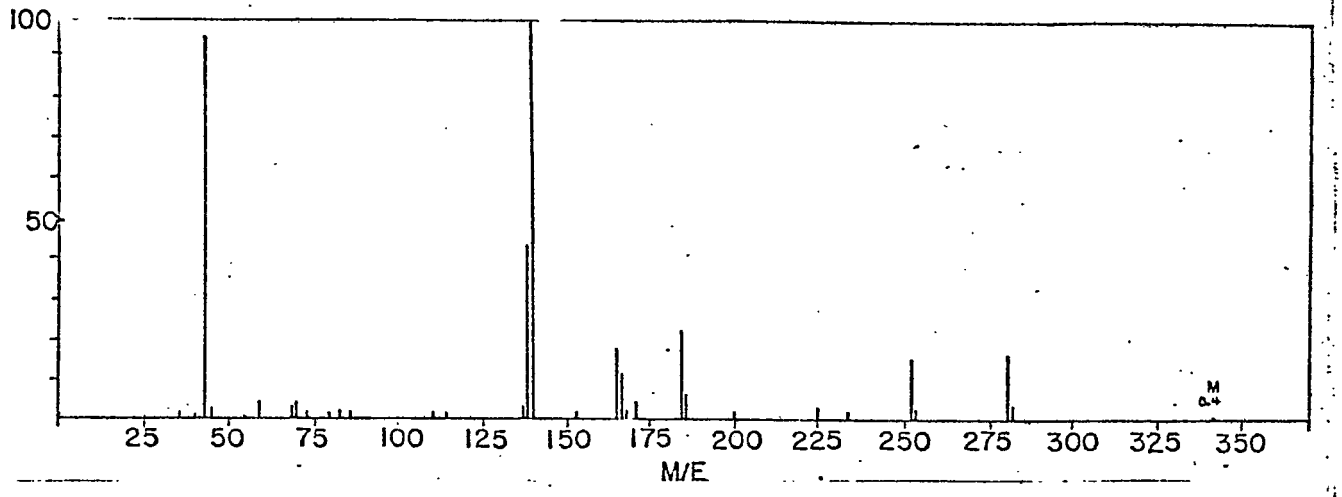

β—AMINOISOBUTYRIC ACID



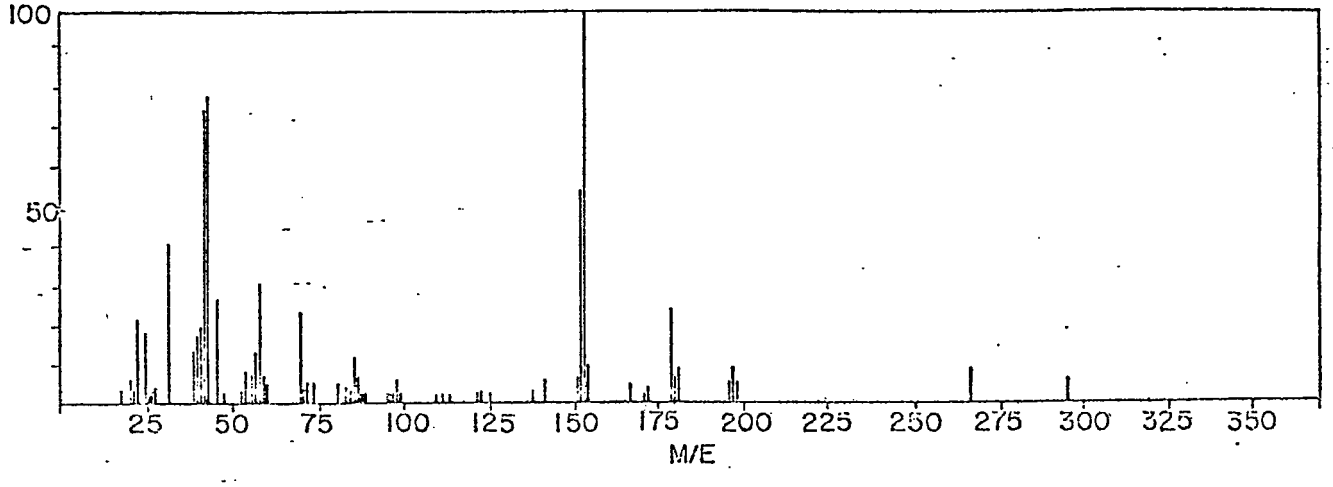
ISOSERINE



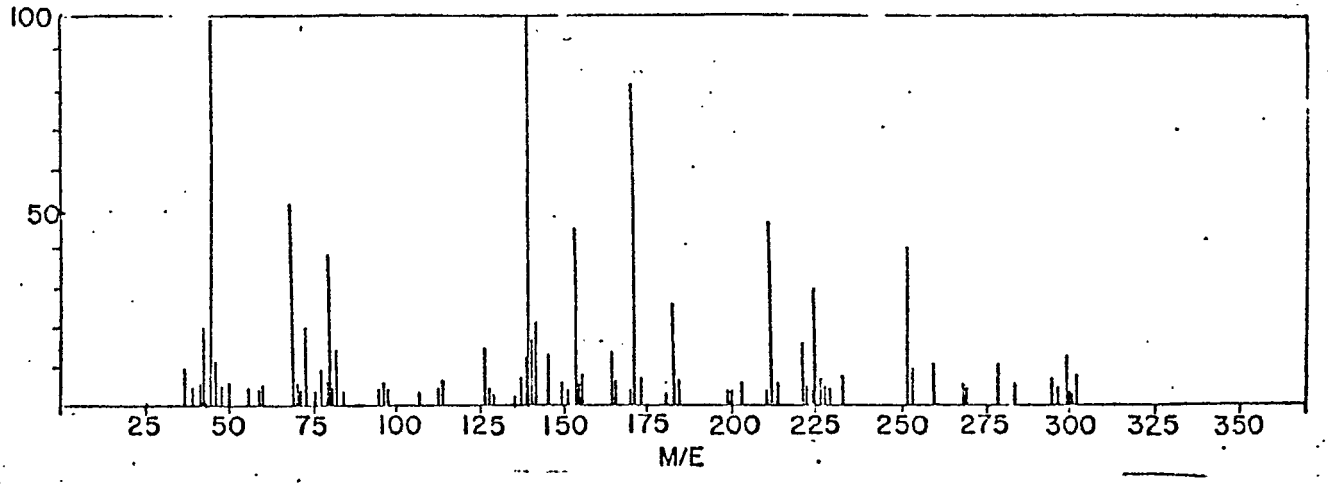

SERINE



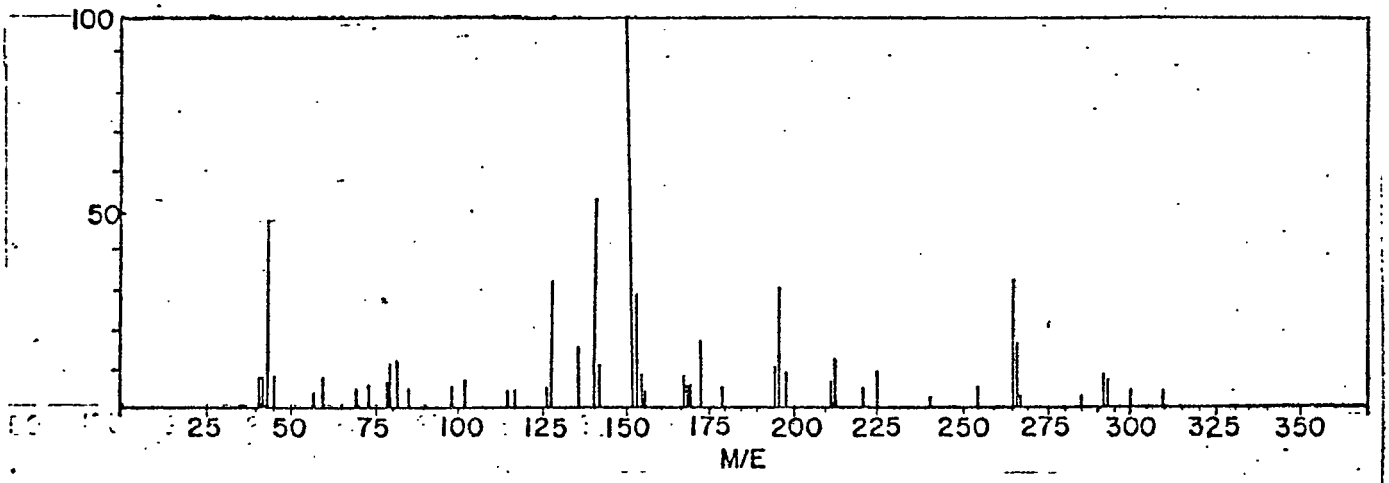

THREONINE



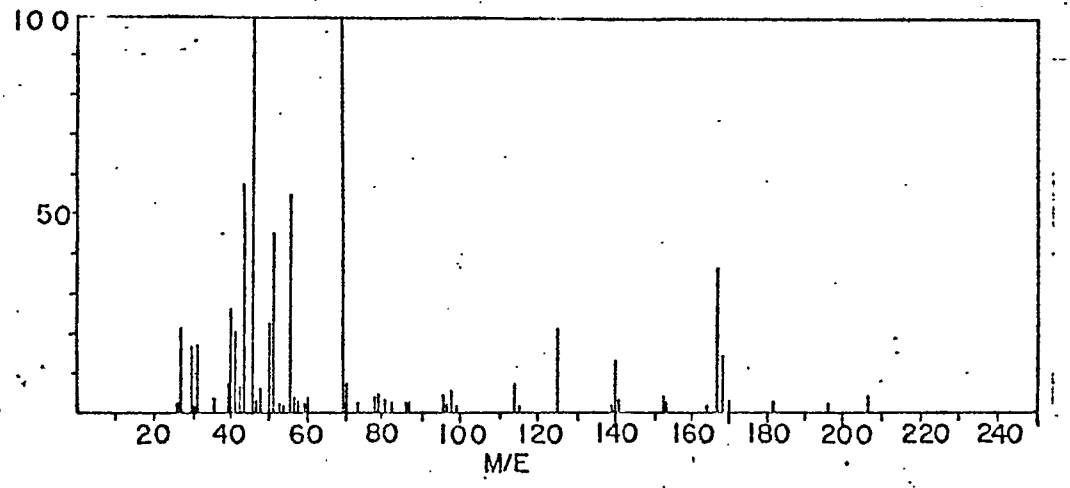
DIAMINOPROPIONIC ACID



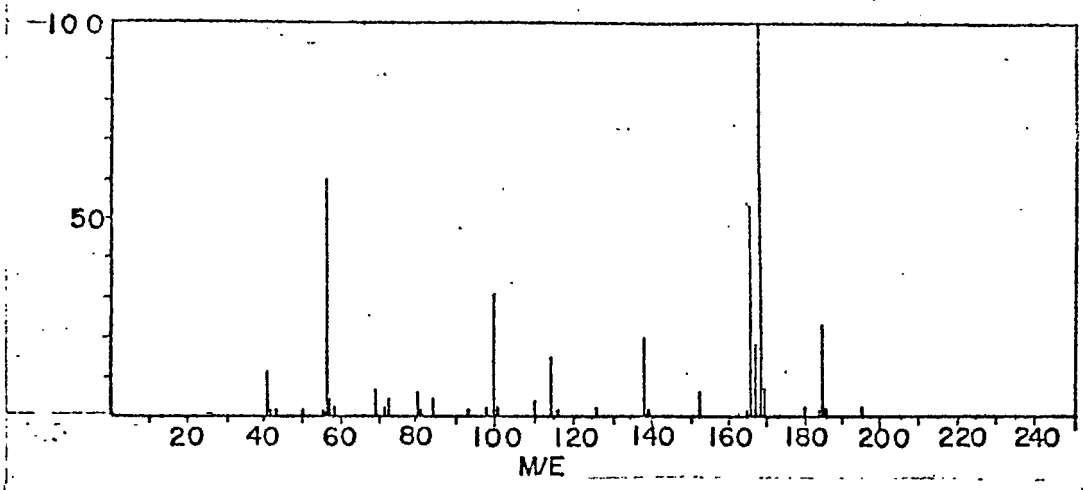

2,4-DIAMINOBUTYRIC ACID



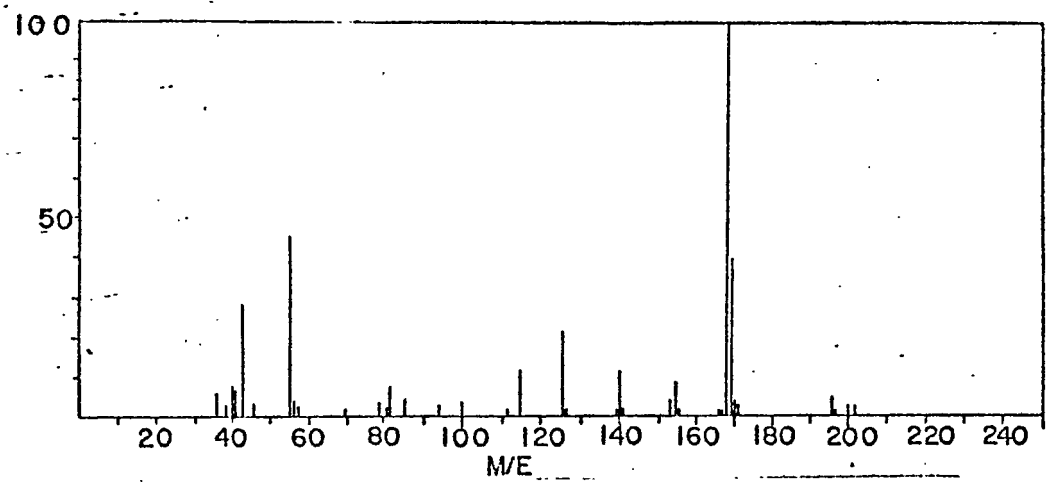

γ-$NH_2$-BUTYRIC ACID



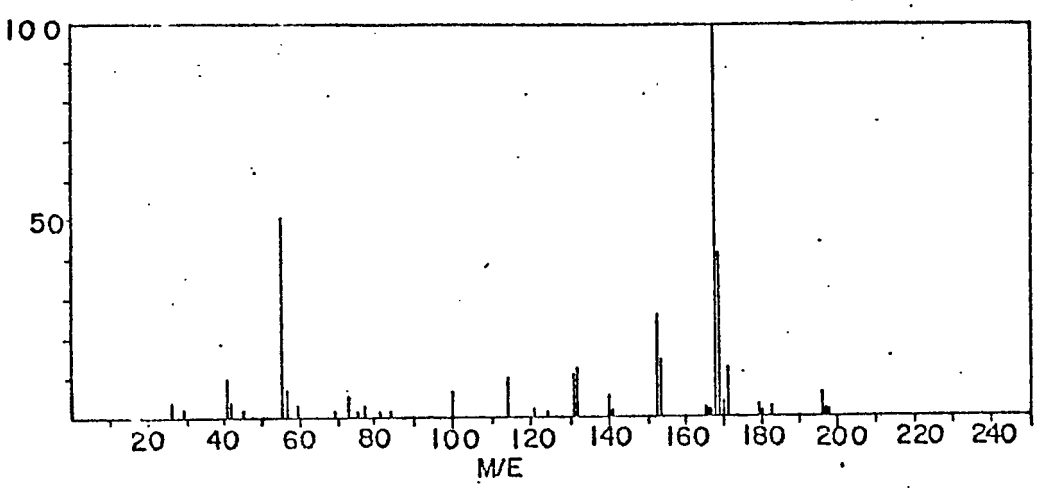
ISOVALINE



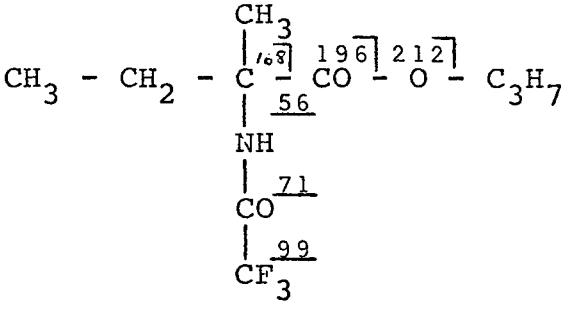

NORVALINE




VALINE



Mass spectral fragmentation of N-TFA isovaline isopropyl ester



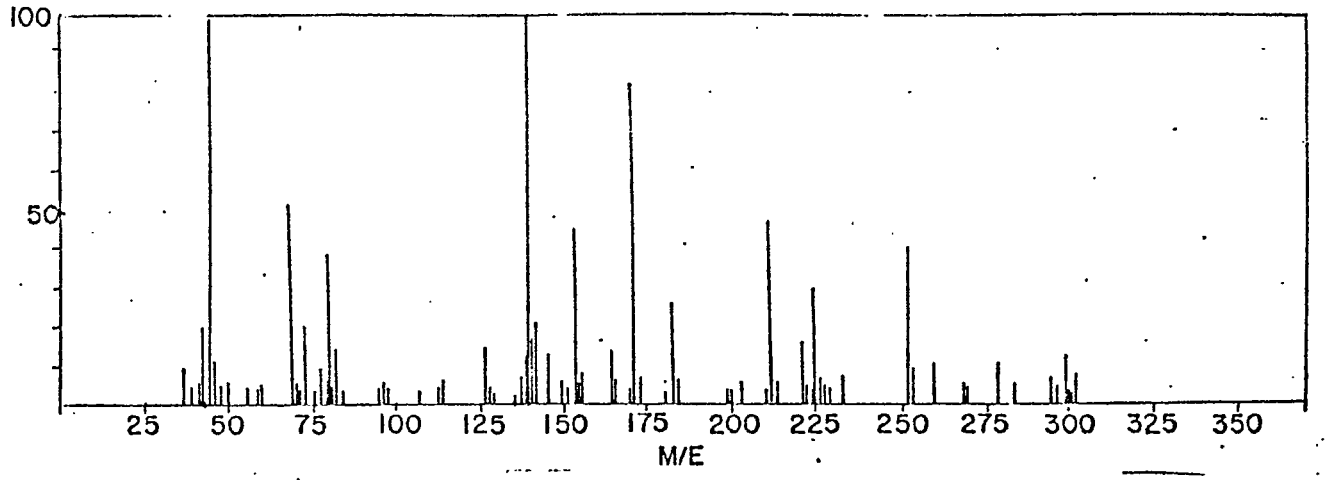

| additional fragments | m/e |
|---|---|
| M - $C_3H_5$ | 213 |
| 212 - CO | 184 |
| 196 - $C_3H_6$ | 152 |
| 168 - $C_3H_6$ | 126 |
| 168 - $2CH_3$ | 138 |

The carboxyalkyl group is always cleaved as the total group. This is indicated by the absence of m/e 43 and m/e 212, and by the formation of m/3 168 as the base peak and its fragments m/3 138 and 126.

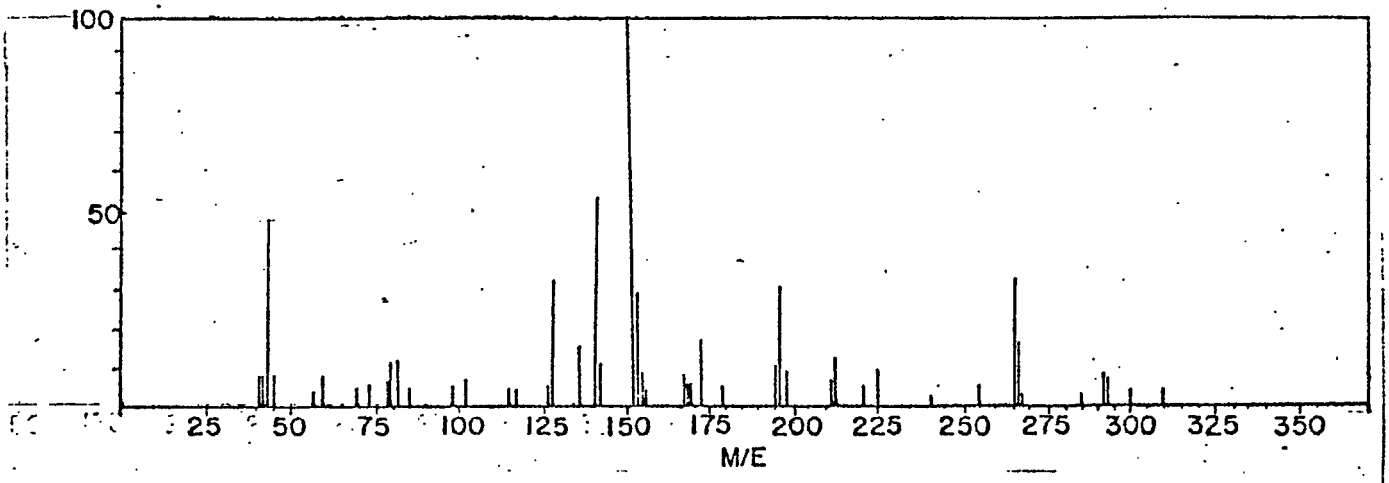
Significant is the high intensity of m/e 56 of the residue molecule without the functional groups. This peak is also characteristic for valine and nonvaline.

The appearance of m/e 138 indicates that cleavage of two methyl groups is easier achieved than in valine, where the methyl group is attached to the β-carbon.

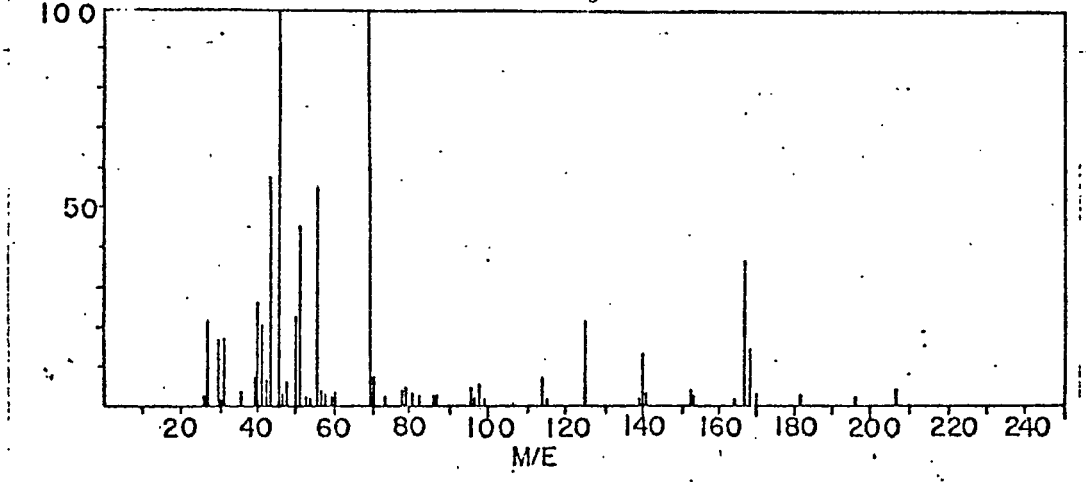
DIAMINOPROPIONIC ACID



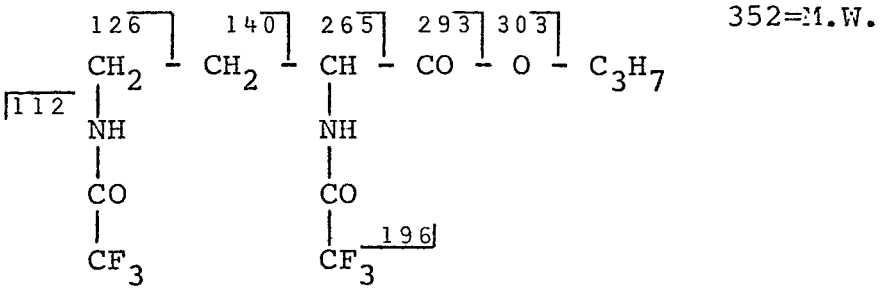

2,4-DIAMINOBUTYRIC ACID




γ-$NH_2$-BUTYRIC ACID

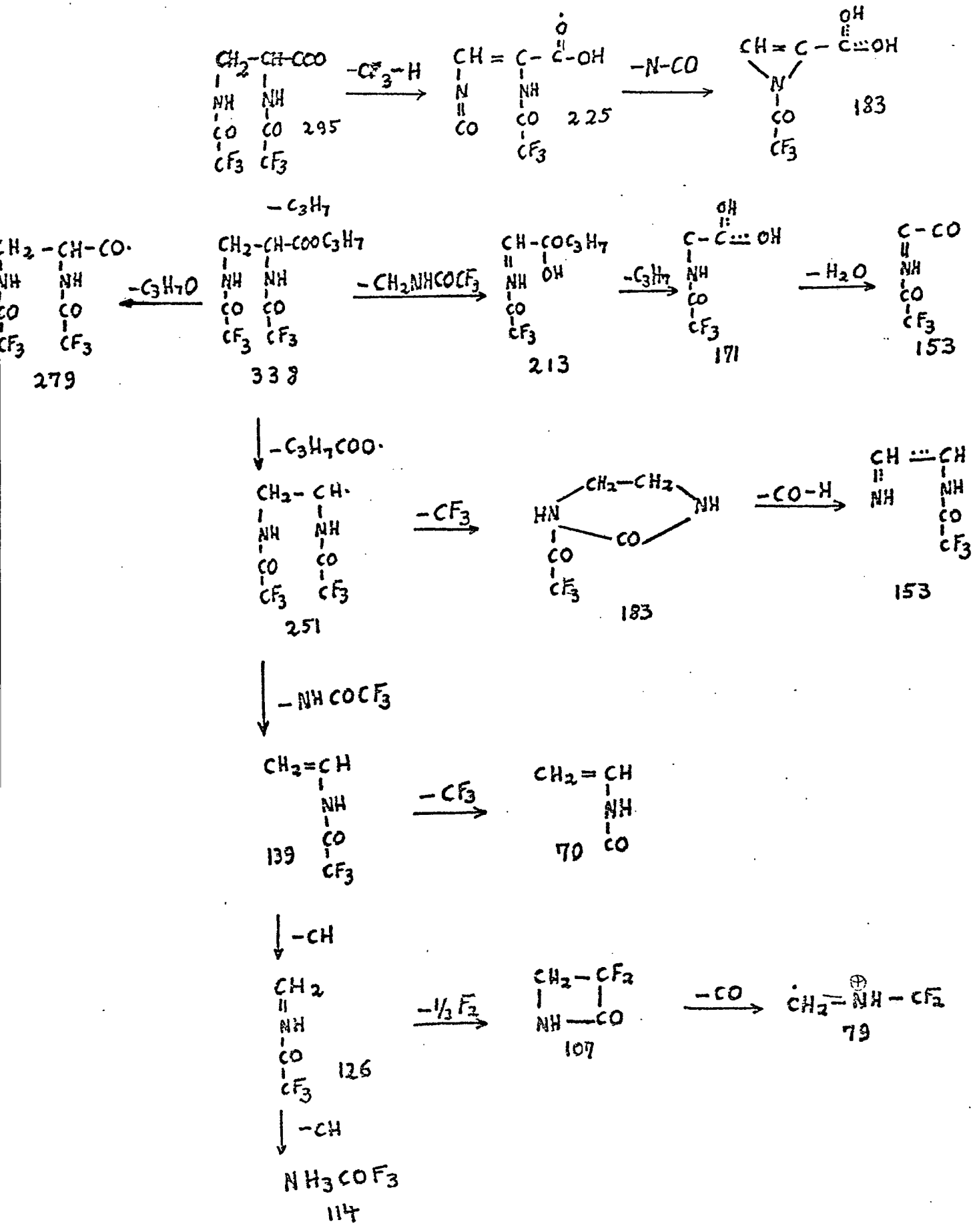


Mass spectral fragmentation of N-TFA-2,4-diaminobutyric acid isopropropyl ester

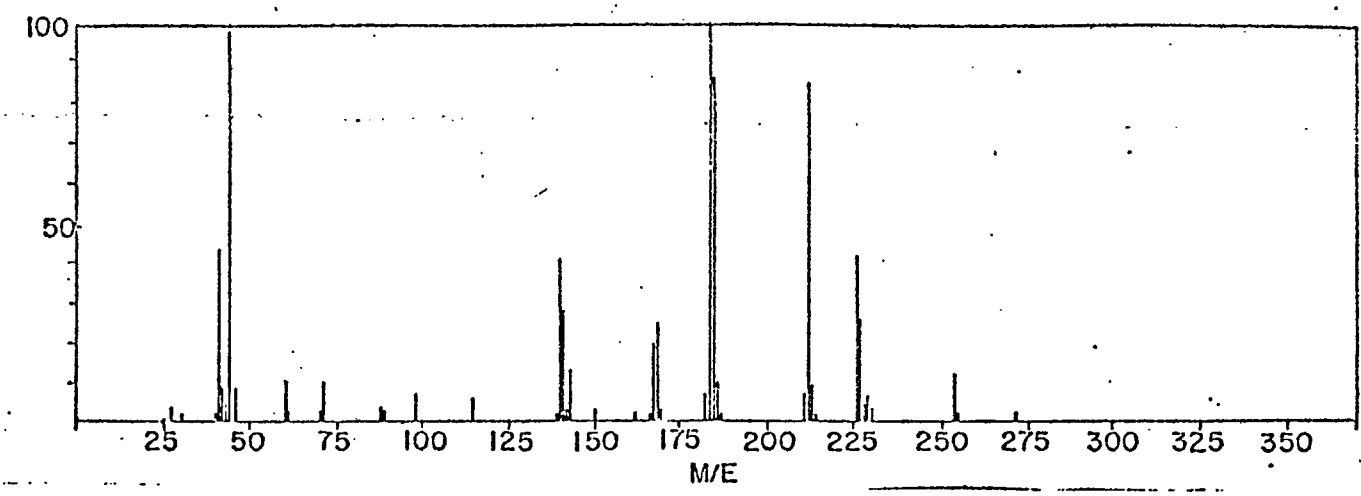



| additional fragments | m/e |
|---|---|
| $293 - CF_3 + H$ | 213 |
| $CF_3CONH\text{-}CH\text{—}CH\text{—}CH_2$ | 152 |

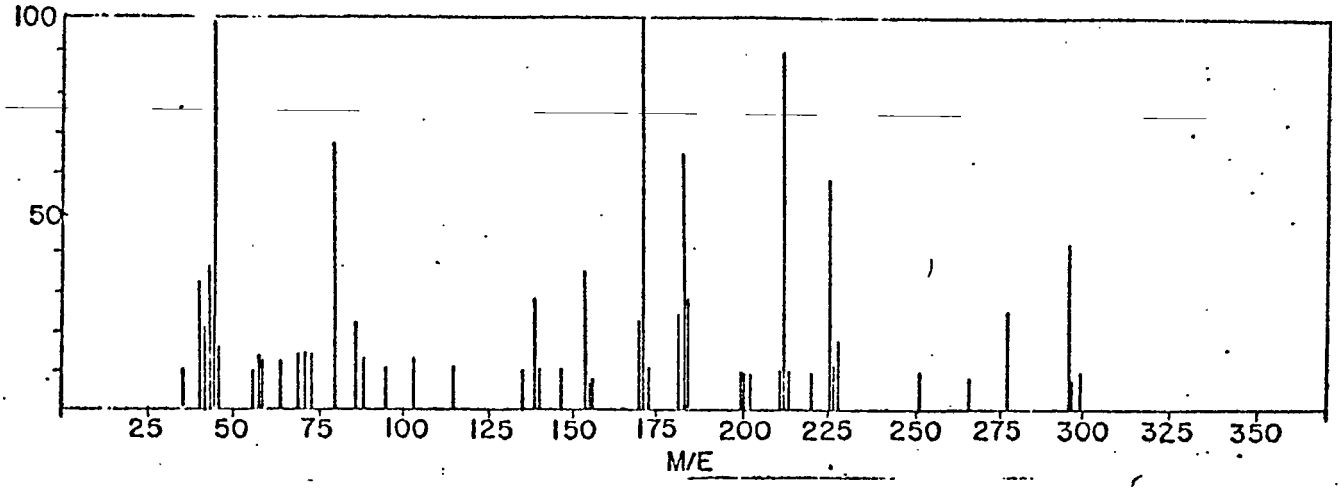
Mass spectral fragmentation pattern of Diaminopropionic acid



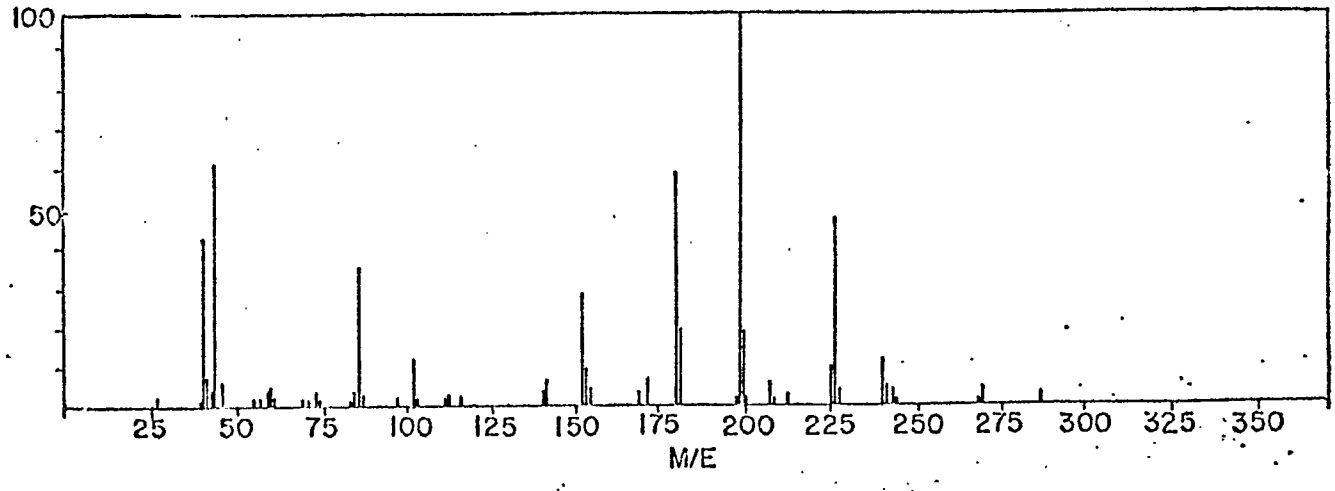
ASPARTIC ACID




DIAMINOSUCCINIC ACID




GLUTAMIC ACID

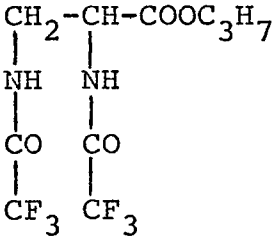


Mass spectral fragmentation of N-TFA-isopropyl esters of diaminopropionic and diaminosuccinic acid

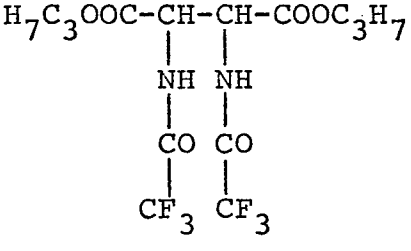
The structural formulas are shown in figure.

| m/e | dap | das |
|---|---|---|
| 251 | 43 | 10 |
| 225 | 32 | 60 |
| 213 | 50 | 90 |
| 183 | 27 | 27 |
| 171 | 80 | 100 |
| 153 | 48 | 35 |
| 138 | 12 | 29 |
| 114 | 8 | 10 |
| 79 | 38 | 68 |
| 69 | 53 | 16 |
| 43 | 99 | 99 |

Das and dap differ by one additional carboxylic acid group in das, which is also



Diaminopropionic acid



Diaminosuccinic acid

estified so that no free acidic groups remain. Both amino acids share a very similar fragmentation pathway as is demonstrated by comparison of their high intensity peaks in the table.

Among the most characteristic ions for dap acid is that corresponding to the loss of the isopropyl carboxy group followed by the abstraction of one of the derivatized amino groups and formation of a five membered ring. Removal of CO from this cyclic system results in a large peak at m/3 153. Interestingly enough the fragmentation of das does not commence in this manner. The first step here is the cleavage of symmetrically structured molecule in halves (m/e 213). Subsequently occurs the loss of the olefin of the ester group resulting in m/e 171 followed by elimination of water producing peak m/e 153.

Amino acids in the fractionated HCN polymerisate:

Nine samples of hydrolysates of fractions of an HCN polymerization product obtained by J. P. Ferris were investigated for amino acids.

1. Acid hydrolysis of acid fraction
2. Base hydrolysis of acid fraction
3. Acid hydrolysis of basic fraction
4. Base hydrolysis of basic fraction
5. Acid hydrolysis of neutral fraction

6. Base hydrolysis of neutral fraction
7. Acid reflux hydrolysis of acid fraction
8. Base reflux hydrolysis of basic fraction
9. Acid reflux hydrolysis of neutral fraction

TABLE 3

The approximate amounts of amino acids in the nine samples [μg]:

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| gly | 1,100 | 1,400 | 45 | 20 | 23 | | 2,000 | 140 | 90 |
| das | 900 | 1,000 | 20 | 2 | | | 1,000 | | |
| glu | | | | | 20 | | | | 140 |
| asp | 70 | 120 | 6 | 8 | 8 | | 41 | 65 | |
| aba | 15 | 15 | | | 7 | | 10 | | 32 |
| α-aib | 15 | 6 | | | 1 | | 1 | | |
| ile | 2 | 12 | | | 4 | | | | |

The significance of the distribution of the amino acids in the nine samples cannot be discussed, because no details on the polymerization experiment are available at the present time.

Anion exchange as well as cation exchange proved to be necessary--even for the hydrolysates of the neutral fraction--in order to avoid the injection of volatile contaminants and salts onto the column. Salts interfere in the preparation

of N-TFA derivatives of amino acids and their subsequent gas chromatography (32). Repeated injections of salt containing samples into the gas chromatograph cause a reduced response. The analysis was performed well aware of the possible sources of contamination (33).

No amino acids were detected in the adsorption filtered and triple distilled water used for ion exchange. The ion exchange separation was carried out with some amino acids as standards. The amino acids were recovered in about 20% yield.

A sample containing glycine, α-aminoisobutyric acid and diaminosuccinic acid in equal amounts was derivatized and chromatographed. The chromatographic peak resulting were in the same order of magnitude.

No amino acids at all were detected in the base hydrolysis of the neutral fraction. The gas chromatographic peaks obtained after cation exchange only did not originate in amino acids. The acid fraction contains amino acids in relatively larger quantities. While diaminosuccinic acid was obtained in the acidic and base fraction, it could not be detected in the neutral fraction--apparently in favor of glutamic acid.

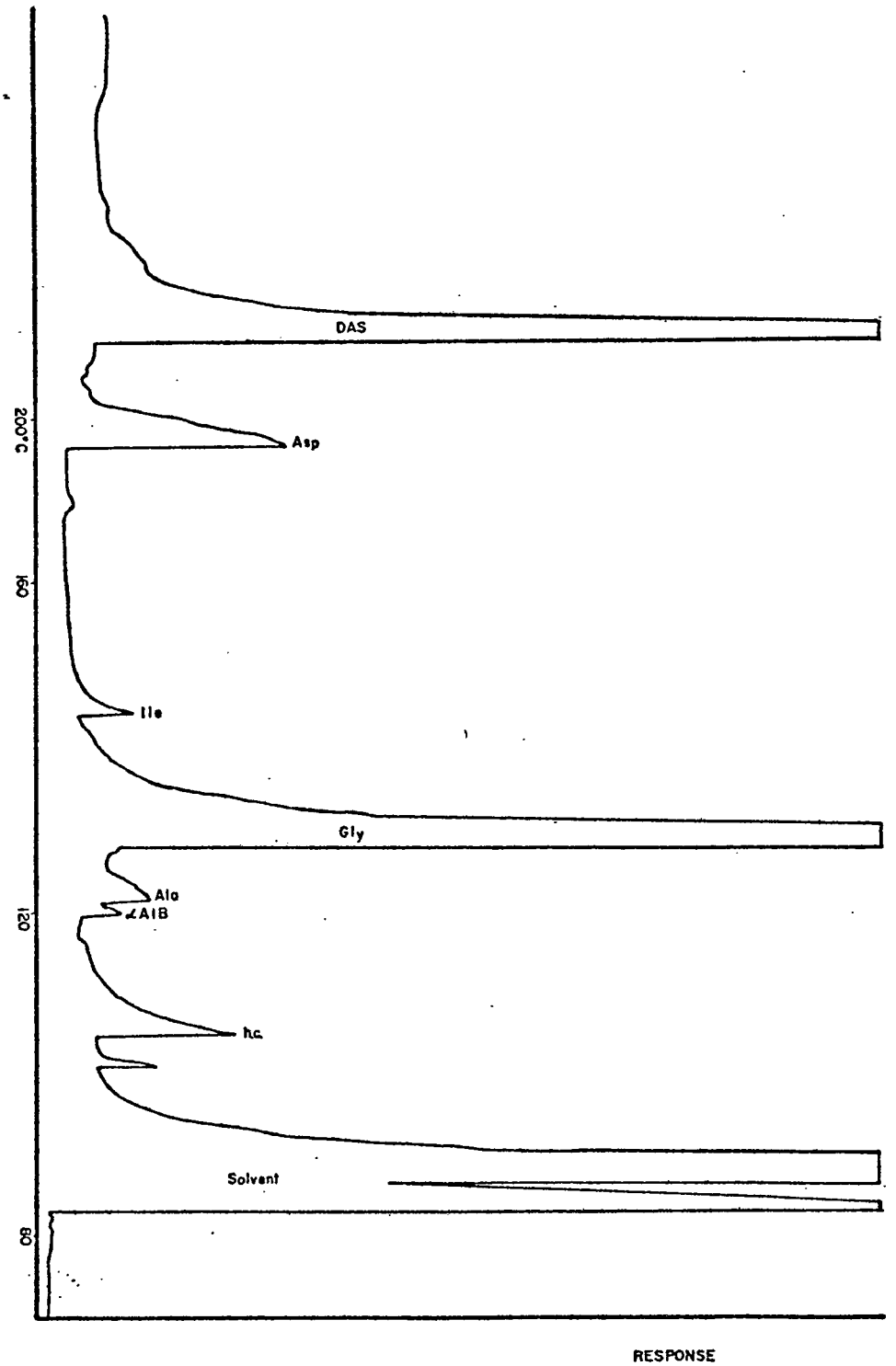
FIGURE 17

Gas chromatogram of N-TFA amino acid isopropyl esters in sample no. 1 of the HCN polymerization experiment.

Column: SF 96 300' x 0.01" I.D.

Conditions: Cryogenic injection; program rate 1° min 70-140° 2° min 140-200° range 4 x $10^{-1}$

Carrier gas: He 4 ml/min


DAS
Asp
Ile
Gly
Ala
AIB
hc
Solvent
200°C
160
120
80
RESPONSE

Summary:

The optimum reaction conditions for the preparation of N-TFA amino acid isopropyl esters was established. The procedure is applicable to the various types of amino acids, 38 protein and nonprotein amino acids as their N-TFA isopropyl derivatives could be elected from a stainless steel capillary column coated with SF-96. For four of the protein amino acids, a less polar stationary phase, OV 101 on chromosorb Q packed in a 1.5 m glass column, had to be used.

The mass spectra of 39 protein and nonprotein amino acids were recorded using a GC-MS combination. The mass spectra were standardized for the use as references.

Amino acids in hydrolyzed fractions of an HCN polymerization product were semiquantitatively determined. The amino acids were isolated by ion exchange, derivatized and chromatographed according to the established procedure. The amino acids were identified by comparing their mass spectra to the standardized reference spectra.

# IV.

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