Quantitative Gas Chromatography of Amino Acids

Preparation of *n*-Butyl *N*-Trifluoroacetyl Esters

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▶ **A procedure was developed for preparing *n*-butyl *N*-trifluoroacetyl esters of amino acids for use in analysis by gas liquid chromatography. This derivative was found to have advantages over others investigated. Single chromatographic peaks were obtained for all of the common protein amino acids except tryptophan and arginine. Tryptophan gave two peaks but could be converted into a single derivative by longer acylation. Reproducibility of response was found to be good for derivatives carried through the entire chemical and chromatographic procedure, and no problems were indicated in the analysis of mixtures. Under anhydrous conditions the derivatives were stable with respect to time, and yield of derivative was found to be above 96% for each of six representative amino acids.**

Because of its speed, accuracy, and sensitivity, gas chromatography offers advantages over other chromatographic methods which have been employed for the determination of amino acids. However, the development of satisfactory gas chromatographic procedures has been rather slow as a result of the fact that amino acids are not sufficiently volatile to permit direct analysis and must thus be converted into volatile derivatives prior to gas chromatography. The present study was undertaken to evaluate the problems involved, and reaction conditions required, in quantitatively converting amino acids into volatile derivatives suitable for analysis by gas liquid chromatography.

Since 1956, a number of derivatives have been investigated for possible use in the gas chromatographic determination of amino acids. Hunter, Dimick, and Corse (*8*) used oxidation with ninhydrin to prepare volatile aldehydes from several aliphatic amino acids, and the procedure was developed by Zlatkis, Oró, and Kimball (*24*) into a fully automated method for the analysis of seven amino acids. Other approaches have included decarboxylation to give volatile amines (*2*), conversion to methyl α-hydroxy esters (*12, 20*), and conversion to methyl α-chloro esters (*13*). In 1957, Bayer, Reuther, and Born (*1*) reported that the methyl esters of a number of aliphatic amino acids prepared by the Fischer method could be separated by gas chromatography. Saroff, Karmen, and Healy (*18*) successfully chromatographed the ester hydrochlorides of 14 amino acids by adding ammonia to the carrier gas, and Nicholls, Makisumi, and Saroff (*14*) explored the possibility of chromatographing the acid salts of amino acid methyl esters by operating the flash heater at a temperature sufficiently high to cause dissociation to the free ester.

One of the first to recognize the advantages of *N*-substituted esters of amino acids as derivatives for gas chromatography was Youngs (*23*), who in 1959 reported that chromatographic peaks had been obtained for the *n*-butyl *N*-acetyl esters of glycine, alanine, valine, leucine, isoleucine, and proline. Johnson, Scott, and Meister (*9, 10*) investigated the preparation and gas chromatographic separation of the *n*-amyl *N*-acetyl esters. Chromatographic peaks were obtained for the derivatives of 36 amino acids including 18 of the common protein amino acids, but difficulties were experienced with tryptophan, histidine, cystine, and arginine. A significant aspect of the work was the use of columns containing small percentages of liquid phase at relatively low temperatures, and the separations achieved were generally good. Graff, Wein, and Winitz (*6*) considered the *n*-propyl *N*-acetyl esters to be the best amino acid derivatives for gas chromatography because of the ease with which they could be separated, but an examination of the properties of a number of derivatives led Blau and Darbre (*3*) to favor the *n*-amyl *N*-trifluoroacetyl esters. Zomzely, Marco, and Emery (*25*) reported that the *n*-butyl *N*-trifluoroacetyl esters of 22 naturally occurring amino acids could be prepared and that they could be chromatographed on a single column by means of temperature programming. Chromatographic separation of the derivatives was fairly complete, but a quantitative study of the procedure used for derivative preparation was not made. Pisano, Vanden Heuvel, and Horning (*15*) investigated the gas chromatographic behavior of the phenylthiohydantoins and methyl 2,4-dinitrophenyl esters, and the latter derivatives were also studied by Landowne and Lipsky (*11*), who obtained high sensitivity by employing an electron capture detector. Rühlmann and Giesecke (*16*) separated a mixture of trimethylsilyl *N*-trimethylsilyl amino acid esters, but the derivatives were not found to be sufficiently stable to be utilized for quantitative analysis (*5*).

The methyl *N*-trifluoroacetyl esters have been investigated by a number of workers including Weygand *et al.* (*22*), Saroff and Karmen (*17*), Wagner and Winkler (*21*), and Hagen and Black (*7*). Cruickshank and Sheehan (*4*) chromatographed all of the common protein amino acids and ornithine after conversion to their methyl *N*-trifluoroacetyl esters, but a detailed study was not made of the quantitative aspects of derivative preparation.

Since most of the functional groups likely to be encountered in any amino acid analysis are also found in the protein amino acids, the present study was limited chiefly to those amino acids which are commonly found in proteins. Emphasis was placed on quantitative and reproducible derivative preparation because it was recognized that a high yield of derivative was an essential prerequisite for an accurate gas chromatographic method for amino acids.

EXPERIMENTAL

Apparatus. A Barber-Colman Model 10 gas chromatograph having a 20-mc. Sr^{90} argon ionization detector (5-ml. internal volume) was used in early phases of the work. Samples were introduced through the sample injection system of an attached capillary column conversion kit, but the included sample splitter was removed. Argon was used as the carrier gas.

Also employed was an F & M Model 300 linear programmed temperature gas chromatograph equipped with an F & M Model 1609 flame ionization attachment. The instrument

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was used without modification, but the column oven and detector module of an F & M Model 400 biomedical gas chromatograph was installed for the final passes of the work. Cylinders packed with silica gel and Linde molecular sieve, type 5A, were placed in the hydrogen and air lines to remove water and hydrocarbons. Nitrogen was used as the carrier gas.

During the course of the investigation, it was frequently necessary to heat samples, with magnetic stirring, in a constant-temperature oil bath. Sample flasks containing magnetic stirring bars were placed in an aluminum pan, and the contents were stirred with a Labline Multi-Magnestir (Labline, Inc.) while mineral oil was circulated in the pan and maintained at a constant temperature by means of an Aminco constant-temperature circulating bath (American Instrument Co.). This arrangement made it possible to heat and stir six samples simultaneously.

Samples were introduced for gas chromatography by means of a Hamilton 701N 10.0-μl. syringe, and an Ott compensating polar planimeter calibrated in square inches and accurate to 0.01 square inch was used for the measurement of peak areas. Unless otherwise indicated, a CaLab Model C rotary evaporator (California Laboratory Equipment Co.) was used for all vacuum distillations.

Reagents. All amino acids except cysteine were purchased from Nutritional Biochemicals Corp. and Mann Research Laboratories. Amino acids obtained from Nutritional Biochemicals Corp. had been tested by available recognized techniques to assure highest purity, and those from Mann Research Laboratories were "Mann assayed" chromatographically pure chemicals. Cysteine was obtained from California Corporation for Biochemical Research and was "A grade," which was the highest purity obtainable from this source. Amino acids used in the study were either of the L- or DL-configuration, and no differences were observed between the chromatographic behaviors of derivatives prepared from L- and DL-amino acids.

Anhydrous methanol was a "Fisher certified reagent" obtained from Fisher Scientific Co. To ensure that it was anhydrous, it was dried by treatment with magnesium turnings prior to use. 1-Butanol was a "Baker analyzed reagent" obtained from J. T. Baker Chemical Co., and trifluoroacetic anhydride was an "Eastman grade" chemical produced by Distillation Products Industries. Hydrogen chloride used as esterification and interesterification catalyst was purchased as a compressed gas from The Matheson Co., Inc., and was 99.0% minimum purity.

Procedure. Analytical Preparation of *n*-Butyl *N*-Trifluoroacetyl Esters. The amino acid or mixture (<60 mg.) was placed in a 125-ml. flat-bottom flask, and 10 ml. of anhydrous methanol containing 1.20 ± 0.10 meq. per ml. of anhydrous HCl were added. The flask was then stoppered with a ground-glass stopper; the solution was stirred on a magnetic stirrer for 30 minutes at room temperature; and the methanol was removed by vacuum distillation at 60° ± 1° C. Ten milliliter of 1-butanol containing 1.20 ± 0.10 meq. per ml. of anhydrous HCl were added; the solution was heated for 180 minutes with magnetic stirring in an oil bath at 90° ± 3° C., and the butanol was removed by vacuum distillation at 60° ± 1° C.

n-Butyl ester hydrochloride(s) was (were) then trifluoroacetylated by adding 5.00 ml. of methylene chloride and 0.50 ml. of trifluoroacetic anhydride and stirring for 120 minutes at room temperature on a magnetic stirrer. The trifluoroacetic anhydride and solvent were removed by vacuum distillation at room temperature, and the *n*-butyl *N*-trifluoroacetyl ester(s) was (were) dissolved in anhydrous chloroform prior to gas chromatography. Preparation of derivatives by this procedure required over 5 hours, but since only about 10 minutes of the analyst's time was needed per sample, a number of samples (six with the apparatus employed) could be carried through the procedure simultaneously.

Preparation of Pure *n*-Butyl *N*-Trifluoroacetyl Esters. Pure *n*-butyl *N*-trifluoroacetyl esters of valine, isoleucine, methionine, glutamic acid, tyrosine, and lysine were synthesized for use as reference standards. One (1.000) gram of amino acid and 200 ml. of 1-butanol containing 2.00 ± 0.10 meq. per ml. of anhydrous HCl were placed in a 250-ml. flat-bottom flask, and the solution was heated in an oil bath at 90° ± 3° C. for 180 minutes with magnetic stirring. After removal of the butanol by vacuum distillation at 60° ± 1° C., 50 ml. of 1*M* aqueous Na_2CO_3 were added and the solution was extracted with two 25-ml. portions of methylene chloride (or until extraction of the amino acid ester was complete in the case of tyrosine) in a 250-ml. separatory funnel. The combined methylene chloride extracts were then extracted with 50 ml. of distilled water, placed in a 125-ml. flat-bottom flask, and evaporated to dryness by vacuum distillation at room temperature. Twenty-five additional milliliters of methylene chloride were added, and evaporation was repeated to remove remaining traces of water by azeotropic distillation.

The residue was dissolved in a solution of 25 ml. of *n*-hexane containing 1.0 ml. of anhydrous diethyl ether, and the solution was saturated with anhydrous HCl. The solvent was then removed by vacuum distillation at room temperature; 25 ml. of *n*-hexane were added and the solution was allowed to stand for 15 hours at −30° C. If the *n*-butyl ester hydrochloride crystallized, the hexane was removed by filtration, and if crystallization did not occur, the solvent was removed by vacuum distillation at room temperature.

The ester hydrochloride was then trifluoroacetylated in a 125-ml. flat-bottom flask by adding 10 ml. of methylene chloride and 3.00 ml. of trifluoroacetic anhydride and stirring for 180 minutes at room temperature on a magnetic stirrer. The anhydride and solvent were removed by vacuum distillation at room temperature, and the *n*-butyl *N*-trifluoroacetyl ester was purified by vacuum distillation. A 10-ml. heart-shaped Claissen flask was employed to purify the derivatives of valine, isoleucine, methionine, and glutamic acid. For tyrosine and lysine, a special molecular distillation apparatus was required (*19*).

Yield of Derivative. Ten milligrams of amino acid were placed in a 125-ml. flat-bottom flask, and *n*-butyl *N*-trifluoroacetyl ester was prepared by the analytical procedure. Approximately the same number of moles of pure *n*-butyl *N*-trifluoroacetyl ester was weighed and placed in another flask, and each derivative (analytical and pure) was dissolved in 2.000 ml. of a 10.0-ml. chloroform solution containing the *n*-butyl *N*-trifluoroacetyl ester prepared from 50.0 mg. of glutamic or aspartic acid. Each solution was then analyzed by gas chromatography under the conditions indicated for Figure 4, and per cent conversion of amino acid to derivative was computed by comparing the peak area obtained for the derivative prepared by the analytical procedure with that obtained for the pure derivative. The butyl trifluoroacetyl ester of glutamic or aspartic acid added to each sample was employed as an internal standard to correct for variations in injected sample volume, solution volume, and instrumental response.

RESULTS AND DISCUSSION

Selection of Derivative. Procedures investigated in preliminary experiments included oxidation to volatile aldehydes with ninhydrin, reduction to amino alcohols with lithium aluminum hydride, preparation of the methyl esters, and preparation of the *n*-butyl *N*-acetyl esters. Chromatographic peaks were obtained for the *n*-butyl *N*-acetyl esters of all of the common protein amino acids except histidine, arginine, and cystine, and the conclusion was reached that the *N*-acyl esters were ideal derivatives for the gas chromatography of amino acids. By employing the proper reagents and reaction conditions it should be possible to esterify all carboxyl groups and to acylate all amino, hydroxy, phenolic, sulfhydryl, imidazole, and guanido groups, thereby masking the polarity of all groups which would cause difficulty in gas chromatographic separations.

Experiments were conducted to evaluate the problems involved in separating the *n*-butyl *N*-acetyl esters for which chromatographic peaks were obtained, and, as shown by Figure 1, a good separation was achieved within 11 minutes for the derivatives of alanine, valine, glycine, isoleucine, and leucine, which are the first five components to be eluted from most columns. A complete separation was not attempted

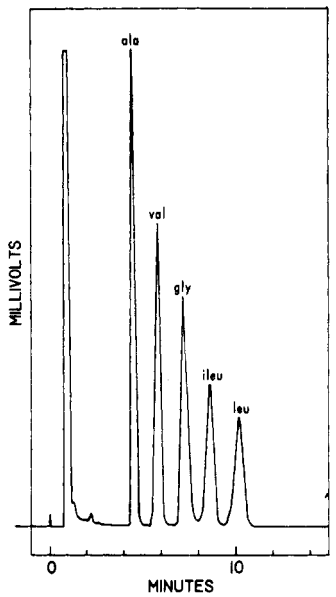


Figure 1. Separation of five *n*-butyl *N*-acetyl amino acid esters (Barber-Colman Model 10)

Column: 250- × 0.47-cm. i.d. stainless steel packed with 0.25% (w./w.) Carbowax 1500 on 30- to 60-mesh acid-washed Chromosorb W. Flow rate: 46 ml. per minute argon. Column temp.: 150° C. Flash-heater temp.: 302° C.

at this time since it was recognized that such a study should be delayed until peaks had been obtained for those amino acids which had given negative results. It was apparent, however, that the desired separations could be attained for any *N*-acyl ester which was sufficiently volatile and sufficiently nonpolar to permit gas chromatography.

In order that their chromatographic behaviors could be compared, the methyl *N*-trifluoroacetyl, methyl *N*-acetyl, *n*-butyl *N*-trifluoroacetyl, and *n*-butyl *N*-acetyl esters of each of the amino acids listed in Table I were prepared and chromatographed. The amino acids were selected to give derivatives which would represent a wide range of volatilities, and the derivatives prepared were chosen to give extremes of volatilities. The temperature of the chromatographic column was programmed as indicated, and the temperature at which each derivative emerged from the column (retention temperature) was recorded. Two different chromatographic columns were employed because the range of volatilities was too great to permit chromatography on a single column.

Table I shows that the trifluoroacetyl esters are more volatile than the corresponding acetyl esters and that the methyl esters are more volatile than the *n*-butyl esters. Table I also reveals that difficulties are involved in the gas chromatography of the acetyl esters of those amino acids which yield the least volatile *N*-acyl esters. The *n*-butyl acetyl ester prepared from lysine tailed badly and was eluted from the chromatographic column at a high temperature, and the methyl acetyl ester was not eluted under the conditions employed. The evidence clearly indicated that the trifluoroacetyl esters were better derivatives than the acetyl esters.

However, the results did not permit a choice between the methyl and *n*-butyl trifluoroacetyl esters. The methyl trifluoroacetyl esters of valine, phenylalanine, and glutamic acid had retention temperatures considerably lower than those for the corresponding *n*-butyl trifluoroacetyl esters, but the methyl and *n*-butyl trifluoroacetyl esters of lysine were eluted at approximately the same temperature. The conclusion was reached that the difficulties involved in the gas chromatographic separation of the methyl or *n*-butyl trifluoroacetyl esters would be approximately equal, and the chromatographic behavior of the ethyl, propyl, and amyl trifluoroacetyl esters would be expected to be similar to that of the methyl and butyl trifluoroacetyl derivatives.

Another important consideration in selecting a trifluoroacetyl ester for gas chromatography is volatility. If volatility is too great, the esters cannot be readily concentrated following trifluoroacetylation, and the analysis of samples containing small amounts of amino acids presents considerable difficulty. It is therefore important to select a trifluoroacetyl ester which can be readily concentrated without volatility losses.

For a comparison of their relative volatilities, the derivatives listed in Table II were prepared from 10.0-mg. quantities of amino acid, chromatographed without removal of the acylation solvent (1.000 ml. of trifluoroacetic anhydride dissolved in 4.000 ml. of methylene chloride), evaporated just to dryness in a rotary evaporator, dissolved in 5.000 ml. of methylene chloride, and then rechromatographed. Because of the accuracy required, 10.0 mg. of glutamic acid hydrochloride were included with the amino acids from which the *n*-butyl *N*-trifluoroacetyl esters were prepared so that the *n*-butyl *N*-trifluoroacetyl ester served as an internal standard.

Table II shows that serious losses were involved in concentrating the methyl trifluoroacetyl ester of valine, and similar losses were observed for other methyl trifluoroacetyl esters. No such difficulties were involved, however, with the *n*-butyl trifluoroacetyl esters, and recoveries of 99, 100, and 97% were obtained for valine, alanine, and glycine, respectively, which would be expected to yield the most volatile derivatives. The *n*-butyl *N*-trifluoroacetyl esters were

Table I. Retention Temperatures of Four *N*-Acyl Esters Prepared from Each of Four Amino Acids

	Retention temperature of derivative,[a] °C.				
	Methyl *N*-trifluoroacetyl	Methyl *N*-acetyl	*n*-Butyl *N*-trifluoroacetyl		*n*-Butyl *N*-acetyl
Amino acid	A[b]	A[b]	A[b]	B[c]	B[c]
Valine	56	65	109	71	110
Phenylalanine	133	141	167	130	156
Glutamic acid	123	137	180	144	170
Lysine	200	[d]	209	173	216 (tailed)

[a] Five microliters of a 2.00-ml. $CHCl_3$ solution containing derivative prepared from 10.0 mg. of amino acid were injected directly on the chromatographic column without the use of a flash heater.
[b] Instrument: F & M Model 400. Column: 100- × 0.3-cm. i.d. borosilicate glass packed with 2.00% (w./w.) neopentylglycol succinate on 80- to 100-mesh acid-washed Chromosorb W. Column temp.: 41° C. for 3.0 minutes, then programmed at 3.3° C. per minute to 218° C., then isothermal for 15 minutes. Other conditions as described for Figure 4.
[c] Conditions of chromatography as described for Figure 4.
[d] Not eluted under the chromatographic conditions employed.

Table II. Volatilities of Methyl and *n*-Butyl *N*-Trifluoroacetyl Esters of Amino Acids

	Peak area, sq. in.		
Derivative	Before evaporation	After evaporation[a]	Recovery, %
Methyl *N*-trifluoroacetyl valine	0.495[b]	0.316[b]	64
n-Butyl *N*-trifluoroacetyl valine	1.255	1.243[c]	99
n-Butyl *N*-trifluoroacetyl alanine	1.71	1.708[c]	100
n-Butyl *N*-trifluoroacetyl glycine	1.37	1.327[c]	97

[a] At room temperature (26° C.) and about 4 mm. of mercury.
[b] Average of four independent determinations.
[c] Corrected with internal standard.

therefore selected for subsequent study because they represented a satisfactory compromise between volatility and ease of chromatographic separation.

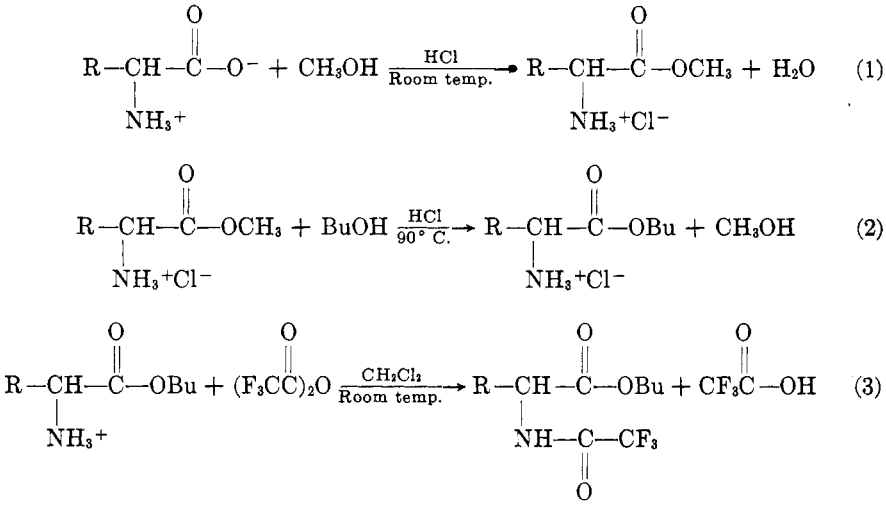
Stability of *n*-Butyl *N*-Trifluoroacetyl Esters. In early experiments with the *n*-butyl *N*-trifluoroacetyl esters, extraneous peaks were often encountered which had not been observed for the corresponding *n*-butyl *N*-acetyl esters. These peaks were found to be the result of decomposition of derivative in the metal flash heater of the chromatographic apparatus and could be reduced in size by lowering the flash-heater temperature. Figure 2 shows chromatograms obtained by injecting 5.00 μl. of a 2.00-ml. chloroform solution of the *n*-butyl *N*-trifluoroacetyl ester prepared from 10.0 mg. of threonine. By lowering the temperature of the flash heater from 223° C. to 142° C., the size of the major extraneous peak was considerably reduced. Problems due to thermal instability were completely avoided by eliminating the flash heater, and no extraneous peaks due to thermal decomposition were observed with any of the derivatives when samples were injected directly on the chromatographic column without the use of a flash heater.

Stability with respect to time was also found to be good. As shown by Table III, recovery, as determined by peak area, was found to be essentially quantitative for derivatives which had been allowed to stand for 90 hours or longer, and no difficulties due to instability were indicated.

When the *n*-butyl *N*-trifluoroacetyl ester of tyrosine, serine, threonine, or hydroxyproline was allowed to stand prior to chromatography, a second peak was often observed which had a longer retention time than the peak initially obtained. The second peak could be made to disappear by reacylation and was apparently due to the monoacyl ester produced by hydrolysis of the trifluoroacetylated phenolic or hydroxy group. Problems due to hydrolysis could be avoided by storing the derivatives in a solvent containing a small amount of trifluoroacetic anhydride.

Preparation of the *n*-Butyl *N*-Trifluoroacetyl Esters. Reaction conditions required to prepare the *n*-butyl *N*-trifluoroacetyl esters were studied, and a procedure for their quantitative preparation was evolved. Esterification presented problems because of the insolubility of cystine and the basic amino acids in butanol. Among the procedures tried were direct esterification with HCl, HBr, and *p*-toluenesulfonic acid as catalysts; direct esterification in a polar solvent; and interesterification with HCl, H_2SO_4, Dowex 50, BCl_3, and BF_3 as catalysts.

The methyl esters, which are more soluble in butanol than the free acids, were easily prepared as a result of the high polarity of methanol, and good results were obtained by methyl ester formation followed by interesterification. The *n*-butyl esters were prepared by esterification in methanol (Equation 1) at room temperature followed by interesterification in 1-butanol (Equation 2) at 90° C. with anhydrous HCl used as a catalyst in both cases; the *n*-butyl ester hydrochlorides were then trifluoroacetylated (Equation 3) at room temperature in a methylene chloride solution of trifluoroacetic anhydride. For cysteine it was necessary to conduct the esterification and interesterification under a nitrogen atmosphere to prevent oxidation.



The time required for interesterification was determined by observing the time required to cause disappearance of the peaks corresponding to the methyl *N*-trifluoroacetyl esters, and a temperature of 90° C. was selected because this was the lowest temperature at which interesterification could be accomplished within 3 hours. The reaction of HCl with butanol to yield butyl chloride and water was already significant at 90° C., and thus a temperature above 90° C. was not employed. The reaction was much slower at 80° C. and almost insignificant at 70° C., but interesterification was not complete at these temperatures.

Because the phenolic group is difficult to acylate under acidic conditions, tyrosine was selected for a study of the time required for trifluoroacetylation. The ratio of the peak area obtained for the *n*-butyl *N,O*-ditrifluoroacetyl ester prepared from 10.0 mg. of tyrosine divided by the peak area of dibutyl suberate used as an internal standard is plotted as a function of trifluoroacetylation time in Figure 3. Trifluoroacetylation was essentially complete after 1 hour, and the conclusion was reached that the *n*-butyl ester hydrochlorides of all of the common protein

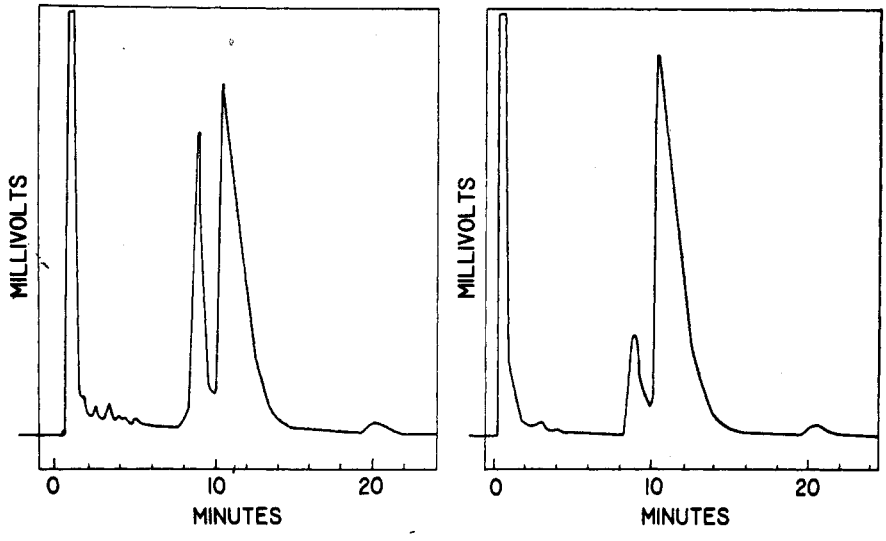


Figure 2. Thermal decomposition of the n-butyl N-trifluoroacetyl ester of threonine as influenced by flash-heater temperature (F & M Model 1609)

Column: 100- × 0.39-cm. i.d. stainless steel packed with 1.00% (w./w.) neopentylglycol succinate on 60- to 80-mesh Gas-Chrom A. Flow rate: 37 ml. per minute N_2. Column temp.: 93° C. Flash-heater temp.: 223° C. left, 142° C. right

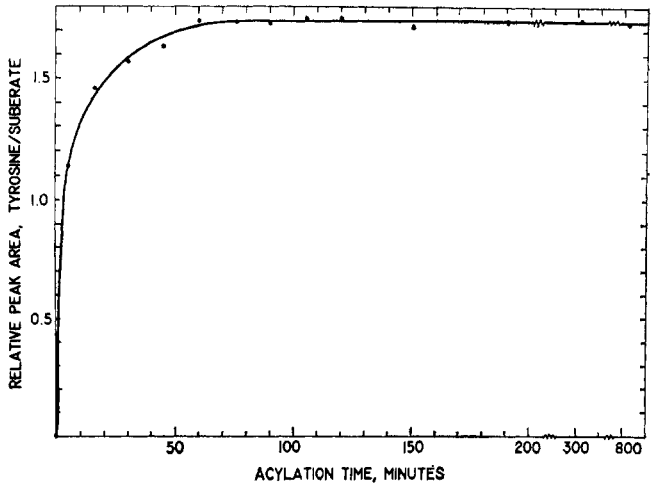


Figure 3. Relative peak area of tyrosine as a function of trifluoroacetylation time

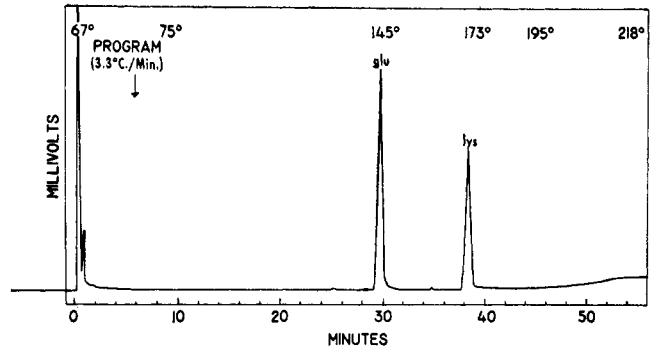


Figure 4. Chromatogram of the *n*-butyl *N*-trifluoroacetyl ester prepared from lysine with glutamic acid as an internal standard (F and M Model 400)

Column: 100- × 0.3-cm. i.d. borosilicate glass packed with 1.00% (w./w.) neopentylglycol succinate on 60- to 80-mesh Gas-Chrom A. Flow rate: 38 ml. per minute N_2. Column temp.: 67° C. for 6.0 minutes, then programmed at 3.3° C. per minute to 218° C. Amplifier range: 100. Sensitivity: 1/128 max. Sample size: 5.0 μl. of a 2.00-ml. $CHCl_3$ solution containing derivative prepared from 10.0 mg. of each amino acid

amino acids, with the exception of refractory ones such as arginine and tryptophan, could be quantitatively trifluoroacetylated by allowing 2 hours for acylation.

By employing the chromatographic conditions described for Figure 4, which is a reproduction of the chromatogram obtained for the lysine derivative, single chromatographic peaks were obtained for all of the common protein amino acids except tryptophan and arginine, and the retention temperatures are recorded in Table IV. Tryptophan gave two peaks apparently because the acylation conditions were sufficiently vigorous to give some diacyl derivative, and the peak having a retention temperature of 176° C. was considered to represent the ditrifluoroacetyl ester. When the acylation was continued for 12 additional hours, the peak having a retention temperature of 203° C. almost disappeared and the second peak became quite large. Under the acylation conditions employed a minimum of 1 hour was required for the quantitative trifluoroacetylation of tyrosine; thus acylation time could not be reduced sufficiently to give a single peak (monoacyl) for tryptophan.

The *n*-Butyl *N*-Trifluoroacetyl Esters as Derivatives for the Analysis of Amino Acids. Relative molar response, with glutamic acid arbitrarily assigned a value of 1.00, was determined independently for each of the 19 amino acids for which peaks were obtained, and, as shown by the last three columns of Table IV, reproducibility of response for amino acids carried through the entire chemical

Table III. Stability of *n*-Butyl *N*-Trifluoroacetyl Amino Acid Esters[a]

Components in sample	Time standing	Recovery,[b] %
Valine	13 hr., 45 min.	100
Phenylalanine		102
Glutamic acid		101
Lysine		103
Glutamic acid	13 hr., 14 min.	99
Valine	15 hr., 55 min.	100
Phenylalanine		103
Glutamic acid		101
Lysine		98
Valine	92 hr., 55 min.	106
Phenylalanine		103
Glutamic acid		105
Lysine		103
Glutamic acid	87 hr., 26 min.	101
Valine	91 hr., 30 min.	100
Phenylalanine		102
Glutamic acid		99
Lysine		99

[a] Each sample was a 2.00-ml. $CHCl_3$ solution containing derivative prepared from 10.0 mg. of each amino acid indicated, and 10.0 mg. of succinic acid was included so that dibutyl succinate served as an internal standard.

[b] Per cent recovery was determined by comparing the peak area obtained after standing with that obtained immediately after derivative preparation.

Table IV. Retention Temperatures and Relative Molar Responses of *n*-Butyl *N*-Trifluoroacetyl Esters[a]

Amino acid	Retention Temp.,[c] °C.	Retention Time, min.	Relative molar response[b] by flame ionization A	B	Av.
Alanine	68	6.3	0.536	0.514	0.53
Valine	70	6.9	0.692	0.664	0.68
Isoleucine	78	9.3	0.786	0.801	0.79
Glycine	83	10.8	0.428	0.433	0.43
Threonine	86	11.8	0.615	0.633	0.62
Leucine	87	12.1	0.764	0.790	0.78
Proline	97	15.1	0.701	0.740	0.72
Serine	98	15.4	0.531	0.548	0.54
Cysteine	115	20.5	0.523	0.501	0.51
Hydroxyproline	120	22.1	0.755	0.766	0.76
Methionine	126	23.9	0.685	0.707	0.70
Aspartic acid	130	25.1	0.940	0.898	0.92
Phenylalanine	130	25.1	1.127	1.114	1.12
Glutamic acid	145	29.6			1.00[d]
Tyrosine	152	31.8	1.014	1.029	1.02
Lysine	173	38.1	0.886	0.830	0.86
Histidine	197	45.4	0.572	0.553	0.56
Tryptophan	176, 203	39.0, 47.2			1.12[e]
Cystine	211	49.6	0.415	0.410	0.41

[a] Five (5.0) μl. of 2.00-ml. $CHCl_3$ solutions each containing derivatives prepared from 10.0 mg. of an amino acid and 10.0 mg. of glutamic acid were injected directly on the chromatographic column without the use of a flash heater. Each value is an independent observation.

[b] Amino acid molar response = area per mole of amino acid. Relative Molar Response $= \frac{\text{Amino Acid Molar Response}}{\text{Glutamic Acid Molar Response}}$.

[c] Conditions of chromatography as described for Figure 4.

[d] Arbitrarily set at unity.

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

and chromatographic procedure was found to be good.

Also, a preliminary experiment suggested that no problems were involved in the analysis of mixtures. A mixture containing 10.0 mg. of each of six different amino acids was carried through the entire procedure, and the relative molar response was determined for each component of the mixture. Table V shows that the values obtained were essentially the same as those obtained when amino acids were carried through the procedure individually. The components of the mixture were selected so that many different functional groups were represented, and, therefore, no problems due to interactions are expected in the analysis of mixtures.

The most important single factor affecting the accuracy of a gas chromatographic method for the determination of amino acids is the yield of derivative. A low yield would probably result in poor precision and would severely limit the usefulness of the method for quantitative analysis. To assess the accuracy to be expected in an actual analysis, it was therefore necessary to determine the yield of derivative for representative amino acids, and pure reference standards were synthesized for this purpose.

Physical constants determined for the *n*-butyl ester hydrochlorides and *n*-butyl *N*-trifluoroacetyl esters of those amino acids from which reference standards were prepared are summarized in Table VI. The boiling-point or melting-point range obtained for each *n*-butyl *N*-trifluoroacetyl ester prepared for use as a reference standard served as a criterion of purity, and purity was also checked by gas chromatography. Each derivative gave a peak having the correct retention temperature, and no extraneous peaks were observed. All evidence indicated high purity.

The results of carbon, hydrogen, nitrogen, and fluorine analyses of the derivatives prepared for use as reference standards are summarized in Table VII. As expected, glutamic acid was found to be converted into the dibutyl ester, and tyrosine and lysine gave the ditrifluoroacetyl derivatives. It had been anticipated that methionine would be converted into its sulfoxide during preparation of its *N*-acyl ester, and a preliminary experiment showed that the *n*-butyl *N*-trifluoroacetyl ester prepared from methionine sulfoxide had the same retention time as the derivative prepared from methionine. Elemental analyses revealed, however, that the sulfur was not oxidized during derivative preparation. The results indicated that in the analytical procedure all carboxyl groups are esterified and all amino, phenolic, hydroxy, sulfhydryl, and imidazole groups are trifluoroacetylated.

Per cent conversions of six amino acids to their *n*-butyl *N*-trifluoroacetyl esters are recorded in Table VIII. Good results were obtained, and the yield was found to be above 96% in each case. The amino acids were selected so that most of the functional groups present in the protein amino acids were represented, and it therefore follows that amino acids can be analyzed with good accuracy by gas chromatography of their *n*-butyl *N*-trifluoroacetyl esters. Further investigations of the method by Stalling, Gehrke, and Shahrokhi (*19*) have shown that quantitative results were obtained for the other protein

Table V. Relative Molar Responses and Recoveries for Amino Acids in Mixture

Component	Relative molar response: Mixture (X)[a]	Relative molar response: Amino acid (Y)[b]	Recovery,[c] %
Leucine	0.773	0.777	99
Serine	0.533	0.540	99
Methionine	0.718	0.696	103
Glutamic acid	1.00[d]	1.00[d]	100
Tyrosine	0.985	1.022	96
Lysine	0.714	0.715	100

[a] Chromatographic conditions were as described for Figure 4, and relative molar responses were computed as indicated in Table IV.
[b] From Table IV.
[c] % Recovery $= \frac{X}{Y}$ 100.
[d] Arbitrarily set at unity.

Table VI. Physical Constants of *n*-Butyl Ester Hydrochlorides and *n*-Butyl *N*-Trifluoroacetyl Esters

Amino acid	*n*-Butyl ester HCl, m.p., °C.	*n*-Butyl *N*-trifluoroacetyl ester: B.P., °C./mm. Hg	n_D^{20}	M.P., °C.
Valine	59.2–60.0	98.0–99.0/43	1.4110	
Isoleucine	Liq.	94.8–95.3/1.7	1.4158	
Methionine	57.6–59.0	126.0–129.0/18	1.4477	
Glutamic acid	62.7–63.2	159.2–161.0/13	1.4278	
Tyrosine (*19*)	161.8–164.0	70–83[a]/ca. 10^{-3}		96.9–98.0
Lysine (*19*)	Liq.	85–90[a]/ca. 10^{-3}		86.0–87.3

[a] These derivatives were purified by molecular distillation, and the temperature given is the temperature of the oil bath.

Table VIII. Per Cent Conversions of Amino Acids to Their *n*-Butyl *N*-Trifluoroacetyl Esters

Amino acid	Yield of derivative, %: A	B	Av.
Valine	100.2	96.8	99
Isoleucine	96.2	96.4	96
Methionine	99.0	96.2	98
Glutamic acid	95.8	98.0	97
Tyrosine (*19*)	102.3	97.2	100
Lysine (*19*)	98.8	98.7	99

Table VII. Elemental Analyses of *n*-Butyl *N*-Trifluoroacetyl Esters of Amino Acids

Amino acid	Empirical formula	Molecular weight	Calculated, %: C	H	N	F	Found,[a] %: C	H	N	F
Valine	$C_{11}H_{18}NO_4F_3$	269.3	49.07	6.74	5.20	21.17	48.82	6.70	5.48[b]	20.97
Isoleucine	$C_{12}H_{20}NO_3F_3$	283.3	50.88	7.12	4.94	20.12	50.86	6.93	4.88[b]	19.88
Methionine	$C_{11}H_{18}NO_3F_3S$	301.3[c]	43.84[c]	6.02	4.65	18.92	44.11[d]	6.04[d]	4.56[d]	19.98[d]
Glutamic acid	$C_{15}H_{24}NO_5F_3$	355.4[e]	50.70	6.81	3.94	16.04	50.75	6.73	4.02[b]	16.12
Tyrosine (*19*)	$C_{17}H_{17}NO_5F_6$	429.3[f]	47.55	3.99	3.26	26.55	47.64	3.94	3.23[d]	26.27
Lysine (*19*)	$C_{14}H_{20}N_2O_4F_6$	394.3[f]	42.64	5.11	7.10	28.91	42.86	5.30	7.13[d]	28.83

[a] All values represent single determinations unless otherwise indicated.
[b] Average of three independent determinations.
[c] Calculated for the methylsulfide and not the sulfoxide.
[d] Average of two independent determinations.
[e] Calculated for the dibutyl ester.
[f] Calculated for the ditrifluoroacetyl ester.

amino acids with the exception of tryptophan and arginine. Tryptophan can probably be analyzed by establishing a response factor for each of the two derivatives obtained, but the chemistry of arginine will need to be investigated in greater detail.

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Study of the Photoionization Detector for Gas Chromatography

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▶ **A study has been made of the properties of a photoionization detector for gas chromatography. The source of ionization is a beam of high energy photons generated in a glow discharge in an inert atmosphere at reduced pressures. The ultraviolet radiation is of such energy that photoionization of the sample molecules occurs without any accompanying ionization of the carrier gas. The main advantage of this detector over other types of ionization detectors is its inherent selectivity based on differences in ionization potentials among the chemical types in the sample. The detector was constructed of a cell made of Teflon and Pt tubing and electrical leads. In general, as ionization potential decreases, response increases. No response was found for the fixed gases CO_2, SO_2, or H_2O. The halogenated compounds disturb the discharge. The apparent ionization efficiency of the device, which has a noise level of 10^{-12} amp. and a background current of 5×10^{-10} amp., is about 10^{-4}. The linear dynamic range is 10^5. The minimum detectable quantity of propane is 2×10^{-10} gram.**

THE OPERATION of the photoionization detector is based on the specific ionization of some ionizable gas or vapor in the presence of an inert carrier gas by irradiation of the mixture with photons of the appropriate energy. A reduced pressure glow discharge in one of the rare gases, N_2, or H_2, can be used to supply ultraviolet radiation of energy greater than the ionization potential of most organic compounds, but less than that of the common carrier gases.

The application of such a device seems to have been contemplated first by Robinson and Brubaker (*12*), but a practical version of the detector was first described by Lovelock (*7*). Recently, a preliminary study of the characteristics of a photoionization detector has been discussed by Roesler (*13*).

Spectrometry in the vacuum ultraviolet has become a field of considerable interest to molecular and atomic spectroscopists. Much valuable information about the highly excited states and electronic structures of molecules is available from this region of the spectrum (*9*). It has been shown by Watanabe (*16*) that photoionization measurements in the vacuum ultraviolet provide a powerful technique for the determination of ionization potentials (*8*). Subsequent work has been carried out using a mass spectrometer in conjunction with a vacuum ultraviolet monochromator–ion source for the determination of photoionization efficiences and cross sections. These are of interest in studying the upper energy states of molecules (*14*).

Photoionization occurs when an irradiated molecule absorbs a quantum of the appropriate energy and undergoes an electronic transition to an ionized state. The ion current resulting from photoionization sets in very sharply as the energy of the incident photons is increased. For compounds of distinct absorption spectra in the vacuum ultraviolet and favorable transition probabilities, even minimum energy quanta—i.e., those of the threshhold energy—give rise to an ion pair. For example, for aromatic hydrocarbons, as the energy of incident photons is increased, the ionization yield shows a sharp leveling off at the ionization potential (adiabatic ionization potentials are obtained with photoionization). With paraffins, however, the break in the curve is less sharp, which is consistent with the diffuse nature of their absorption spectra in the vacuum ultraviolet (*10*).

Photoionization–mass spectrometric studies on polyatomic organic compounds have shown that the ionization and absorption cross sections for the parent ion increase sharply at the ionization threshhold, pass through a maximum, and fall off as the incident photon energy is increased. However, fragment ions often are formed which have higher appearance potentials than the parent ion and cross sections extending to higher energies. Thus an ionization current may be obtained over a wide energy span.