Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Gehrke, Charles W. and Stalling, David L.(1967) 'Quantitative Analysis of the Twenty Natural Protein Amino Acids by Gas-Liquid Chromatography', Separation Science and Technology, 2: 1, 101 — 138
To link to this Article: DOI: 10.1080/01496396708049919
URL: http://dx.doi.org/10.1080/01496396708049919

SEPARATION SCIENCE, 2(1), 101–138 (1967)

Quantitative Analysis of the Twenty Natural Protein Amino Acids by Gas-Liquid Chromatography*,†,‡

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Summary

A quantitative gas-chromatographic method has been developed and applied to the analysis of the 20 protein amino acids and other nonprotein amino acids as their N-trifluoroacetyl *n*-butyl esters. Pure reference standards were prepared and used to determine yield of derivatives. Yields of 98 ≠ 3% were obtained for all the amino acids. The calibration curves were linear and showed no statistical bias. Results obtained from analysis of amino acids mixtures and proteins by gas chromatography were in excellent agreement with theory and ionexchange. For quantitative analysis the amount of amino acid injected is 0.05 to 35 μg. The relative standard deviations range from 0.66 to 2.28%. Qualitative analyses can be made on 1 to 10 ng of amino acid injected.

The merits and applicability of the relative mole per cent, internal standard, and slope factor methods of calculation of the percentage of amino acids are presented and discussed. This method has broad implications in advancing studies in genetics, protein biochemistry, agricultural sciences, medical research, and nutrition.

Historically, the determination of amino acids in protein hydrolysates and other samples of biological origin has proved to be a very difficult problem for the analytical chemist. Chromatography

* This article will be published later in a volume entitled *Separation Techniques: Proceedings of the Nineteenth Annual Summer Symposium on Analytical Chemistry.*

† Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 5017. Approved by the Director.

‡ Supported in part by grants from the National Science Foundation (G-18722 and GB-1426).

would seem to be the best presently available method for analyzing such a complex mixture, but this approach is not without complications, because the "natural" amino acids do not form a homologous series and thus do not differ from each other in any systematic way. However, investigations by Moore et al. (*21,22,29*), Hamilton (*11–13*), Piez (*24*), and Woods et al. (*37*) have developed chromatography into a refined method for the quantitative determination of amino acids, and today this procedure, or some modification of it, is clearly the method of preference.

The development of a gas-liquid chromatographic procedure would seem to be the next logical step in the evolution of methods for the quantitative determination of amino acids. Gas chromatography offers the obvious advantages inherent in the refined separations possible, small sample sizes required, and in the speed, accuracy, and simplicity of the method. Difficult separations of multicomponent systems are routinely accomplished in a matter of minutes, and analysis time has been reduced to a few seconds in some cases. The entire field of gas chromatography has undergone rapid development in recent years, and a number of very fine commercial instruments are now available.

However, the development of satisfactory quantitative 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. Thus our research program was undertaken to evaluate the problems involved, and exact organic reaction conditions required, in quantitatively converting amino acids into volatile derivatives suitable for analysis by gas-liquid chromatography. This has necessarily involved a study of factors such as yield, volatility, and stability of derivative; general applicability; limit of detection; and ease of chromatographic separation.

A suitable volatile derivative of the amino acids for quantitative gas-chromatographic analysis should meet the following criteria:

1. The derivative should be simple in its formation, no rearrangements or structure alteration.
2. The derivatization reaction should go to 95 to 100% completion.
3. There should be no sample loss on vacuum concentration of the sample solution.

4. The derivative must be stable with respect to time and temperature.

5. The derivative must have increased volatility, thus lower retention time.

6. The derivative must be in a form with little or no reactivity with the solid inert support, and resolvable.

Since 1956, a number of derivatives have been investigated for possible use in the gas-chromatographic determination of amino acids. Hunter et al. (*14*) used oxidation with ninhydrin to prepare volatile aldehydes from several aliphatic amino acids, and the procedure was developed by Zlatkis et al. (*39*) into a fully automated method for the analysis of seven amino acids. Other approaches have included decarboxylation to give volatile amines (*3*), conversion to α-chloro methyl esters (*20*). In 1957, Bayer et al. (*2*) reported that the methyl esters of a number of aliphatic amino acids prepared by the Fischer method could be separated by gas chromatography. Saroff et al. (*28*) successfully chromatographed the ester hydrochlorides of 14 amino acids by adding ammonia to the carrier gas, and Nicholls et al. (*23*) explored the possibility of chromatographing the acid salts of amino acids methyl esters by operating the flash heater at a temperature sufficiently high to cause dissociation of the salt 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 (*38*), who in 1959 reported that chromatographic peaks had been obtained for the N-acetyl *n*-butyl esters of glycine, alanine, valine, leucine, isoleucine, and proline. Johnson et al. (*15,16*) investigated the preparation and gas-chromatographic separation of the N-acetyl *n*-amyl 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 et al. (*9*) considered the N-acetyl *n*-propyl 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 (*4*) to favor the N-trifluoroacetyl

n-amyl esters. Zomzely et al. (*40*) reported that the N-trifluoroacetyl *n*-butyl 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 et al. (*25*) investigated the gas-chromatographic behavior of the phenylthiohydantoins and 2,4-dinitrophenyl methyl esters, and the latter derivatives were also studied by Landowne and Lipsky (*19*), who obtained high sensitivity by employing an electron capture detector. Rühlmann and Giesecke (*26*) separated a mixture of amino acid N-trimethylsilyl trimethylsilyl esters, but the derivatives were not found to be sufficiently stable to be utilized for quantitative analysis (*6*).

The N-trifluoroacetyl methyl esters have been investigated by a number of workers including Weygand et al. (*36*), Saroff and Karmen (*27*), Wagner and Winkler (*35*), and Hagen and Black (*10*). Cruickshank and Sheehan (*5*) chromatographed all the common protein amino acids and ornithine after conversion to their N-trifluoroacetyl methyl esters, but a detailed study was not made of the quantitative aspects of derivative preparation.

During the past five years, Lamkin and Gehrke (*18*) and Gehrke et al. (*7*), Stalling and Gehrke (*31*) and Stalling et al. (*32*) have developed a quantitative single general method for the determination of the 20 natural protein amino acids. Gehrke and Shahrokhi (*8*) also reported on chromatographic separations of the N-trifluoroacetyl *n*-butyl esters of the amino acids.

The N-trifluoroacetyl *n*-butyl ester derivative was the derivative of final choice. In the method, esterification in methanol was followed by the interesterification in *n*-butanol to obviate solubility problems of cystine and the basic amino acids in *n*-butanol. To achieve quantitation of derivative formation for arginine and tryptophan a closed-tube acylation procedure was found necessary.

Our investigations have demonstrated that the protein amino acids, as their N-trifluoroacetyl *n*-butyl esters, can be analyzed quantitatively by gas-liquid chromatography.

This paper reports on details of the derivatization method for amino acid analysis; quantitation, limit of detection, chromatography, and application to amino acid mixtures and protein hydrolysates.

EXPERIMENTAL METHOD

Reagents

Methanol, methylene chloride, and chloroform were "Fisher certified anhydrous reagents." To ensure that the methanol was completely anhydrous, it was dried by treatment with magnesium turnings (Mallinckrodt analytical reagent): 1 liter of methanol with 6.0 g of magnesium turnings was refluxed for 2 hours and then distilled in an all-glass system protected from atmospheric moisture with a $CaCl_2$ drying tube.

All the amino acids except cysteine were purchased from Nutritional Biochemical Corp. and Mann Research Laboratories. Cysteine was obtained from California Corporation for Biochemical Research, and was "A grade."

Anhydrous hydrogen chloride used as esterification and interesterification catalyst was purchased as a gas from the Matheson Co., and was of 99.0% purity.

Methanol and *n*-butanol with anhydrous HCl gas (1.25 meq/ml). Anhydrous HCl gas was bubbled into 100 ml of the alcohol until the solution contained approximately 5.0 g HCl/100 ml. The solution was then titrated with NaOH to the phenolphthalein end point to determine the actual concentration of HCl.

Trifluoroacetic anhydride was "Eastman Grade" chemical produced by Distillation Products Industries. 1-butanol was a "Baker analyzed reagent" obtained from J. T. Baker Chemical Co. and was used as received.

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

Apparatus and Glassware

Interesterification of the amino acids at 90 and 100°C was done using an aluminum pan filled with thermally stabilized oil supported by a Cenco Multiple Magnetic Hot Plate Stirrer (16665). Lead rings were placed over the flask necks to hold them secure.

The oil bath, in which the closed-tube acylation was conducted, consisted of a $3\frac{1}{2}'' \times 4'' \times 6''$ aluminum pan supported on a magnetic stirrer to maintain uniform temperature of the oil bath. Two (2) 100-watt coffee heaters were placed in the pan. Temperature control was achieved by using a Variac.

A super D-21-36 safety shield obtained from Instruments for

Research and Industry (I^2R) was used to provide maximum protection from accidental breakage of the acylation centrifuge tube.

An all-teflon rotary evaporator obtained from California Laboratory Equipment Company (Calif. Lab. Model C rotary evaporator) was used to remove the volatile solvents. The vacuum was produced with a Welch Duo-Seal vacuum pump (W. M. Welch Scientific Co., Chicago, Ill.) having a capacity of 140 liters/min. A Calab "cold-finger" condenser containing dry ice in ethylene glycol monomethyl ether was placed between the evaporator and the vacuum pump to prevent volatile compounds from reaching the pump. A sodium hydroxide trap was placed between the condenser and the vacuum pump to protect it from corrosive acidic compounds.

Acylation tube. Standard Pyrex glass, Corning Glass Works Co. No. 8142, 12 ml, conical, screw-cap heavy-duty graduated centrifuge tube with No. 9998 teflon-lined caps having thread No. GCMI 415-15 was used. A preferable acylation tube was obtained from the Fischer and Porter Co., Warmister, Pa. This tube is a $\frac{3}{8} \times 3$ in. Carius combustion tube No. 320-002-0038.

Syringes. Hamilton 701 N ten (10) microliters, Hamilton Co., Whittier, Calif.

Flat-bottom boiling flask, 125 ml, ℸ 24/40 Catalog No. Corning 4100.

Instrumental and Chromatographic Requirements

Minimum. Single-column instrument with temperature programming, single hydrogen flame-ionization detector, and recorder equipped with disc integrator. Instrument should have glass injection ports or glass injector liners which can be inspected for buildup of deposits and readily replaced and cleaned.

Recommended. Dual-column all-glass system with direct on-column injection, differential flame-ionization detector, recorder equipped with disc integrator, and an automatic multiple-rate temperature programmer.

Early experiments indicated that threonine and arginine were decomposed when injected into a hot metal flash heater. The use of direct on-column injection eliminated problems of derivative thermal breakdown. When glass injectors were used at elevated temperatures, no adverse effects on these or other amino acid derivatives were observed.

The availability of multiple program rates greatly facilitates the chromatographic examination of samples, as the program rate may be systematically varied. The resolution of the derivatives with high retention temperatures is more readily achieved using a more rapid program rate than are the derivatives with low retention temperature.

Instrumental Operating and Chromatographic Conditions Used

Instrument. F and M Model 300 linear programmed-temperature gas chromatograph, and F and M Model 1609 flame ionization attachment, with an F and M Model 400-column oven and detector module.

The chromatograph was equipped with a −0.2 to 1.0 mv Minneapolis-Honeywell Electronik 16 series Y-143 recorder (1-sec full-scale response). The recorder was equipped with a Disc Chart Integrator Model 201. Ten-in. by 1-in. metal cylinders were packed with silica gel and Linde molecular sieve type 5A and placed in the nitrogen, hydrogen, and air lines to remove water and hydrocarbons.

Also, an F and M Model 402-3S dual-column linear programmed-temperature biomedical gas chromatograph was used. It was equipped with a dual-channel three-speed Honeywell Electronik 16 recorder of 1-sec full-scale response and Disc Integrator Model 228.

A Micro Tek Model MT-220 linear programmed-temperature biomedical instrument equipped with a four-column oven bath, with two dual-flame-ionization detectors, Model 739810, and two electrometers, Model 636800, was also used.

The chromatographic conditions used with the packed 0.75/0.25 w/w% DEGS/EGSS-X column on 60–80 mesh acid-washed Chromosorb W were:

Column:

3-mm I.D. × 1-m U-shaped borosilicate glass column.

Conditions:

Column temperature: Initial 67°C, final 218°C	
Delay before program:	6 min
Program rate:	3.3°C/min
Detector cell temperature (at start):	123°C
Sensitivity:	1/32
Carrier flow, N_2:	38 ml/min at 20 psig
Air (to detector):	450 ml/min at 20 psig

Hydrogen (to detector):	36 ml/min at 20 psig
Chart speed:	$\frac{1}{3}$ in./min

For the 5% DC-550 column the conditions were:

Column temperature: Initial 125°C, final 225°C	
Delay before program:	3 min
Program rate:	4°C/min
Detector temperature:	250°C
Carrier flow, N_2:	70 ml/min at 20 psig
Air (to detector):	450 ml/min at 20 psig
Hydrogen (to detector):	45 ml/min at 20 psig

Substrates and Supports. DEGS (diethylene glycol succinate), EGSS-X (ethylene glycol succinate methyl silicone polymer), and DC-550 (methyl silicone polymer) were obtained from Applied Science Laboratories, Inc., Box 140, State College, Pa. Neopentyl glycol sebacate, butane-1,4-diol sebacate, and pentane1,5-diol sebacate obtained from Analabs, Inc., were employed in our more recent separation studies. These substrates were coated at concentrations of 0.5 w/w % on 80–100 mesh acid-washed Chromosorb G and packed in 1.5 m × 4 mm I.D. glass columns.

Chromosorb W, 60/80 mesh non-acid-washed, was obtained from Varian Aerograph, Walnut Creek, Calif. It was treated overnight with concentrated HCl. The Chromosorb W was then washed with distilled water until it showed neutral with litmus paper, and was dried overnight at 100°C. The acid-washed support was regraded before use.

Column Preparation. 9.900 g of 60/80 mesh acid-washed Chromosorb W was placed in a 250-ml fluted flask having a standard-taper (T) 24/40 ground-glass joint. A sufficient amount of chloroform was added to cover the inert phase.

0.025 g of EGSS-X (ethylene glycol succinate methyl silicone polymer) was dissolved in 25 ml of chloroform and transferred quantitatively into the flask, followed by thorough mixing. The solvent was then removed at about 65°C by vacuum distillation with a Calab rotary evaporator. Acetone was then added to cover the prepared material and 0.075 g of DEGS dissolved in 25 ml of acetone was transferred quantitatively into the flask. The mixing and solvent removal were as described above. To ensure complete mixing, the flask was allowed to rotate for 5 min or longer after complete removal of solvent. The column material was packed in a 1.00-m × 4-mm I.D. U-shaped borosilicate-glass column by a

combination of vibration with an electric sander and gentle tapping. Then the column was conditioned for 12 hours or more at a temperature of less than 180°C, with a flow rate of 38 ml N_2/min.

The maximum temperature at which the DEGS may be operated depends on the length of time the column is held at this temperature or upon the source from which the DEGS is obtained. We are using stabilized DEGS (C-6), which can be used as high as 260°C for relatively short periods of time. This material is available from Analabs, Inc., 9 Hobson Avenue, Hamden, Conn. Columns of other substrates were prepared in a similar manner.

The DEGS/EGSS-X column will usually last for a minimum of 35 analyses before changes in separation occur, caused primarily by the breakdown of the EGSS-X.

Protein Sample Preparation

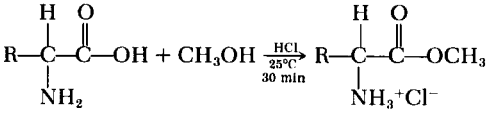
Method I—Protein Hydrolysate. 100.0 mg of protein (dried over P_2O_5 at room temperature to a constant weight) was hydrolyzed with 25 ml of constant boiling HCl (6 *N*) for 20 hours at 100°C in a sealed tube under a nitrogen atmosphere. The sample was then taken to dryness at room temperature on a rotary evaporator. The residue was dissolved in 20.0 ml 0.1 *N* HCl. A 5-ml aliquot of the above hydrolysate was transferred into a ₹ 125-ml flat-bottom flask and shell-frozen, then lyophilized. After lyophilization, one can dry the hydrolysate over P_2O_5 to remove all moisture. The residue was then taken through the derivatization procedure for gas chromatography as described below.

Method II—Protein Hydrolysate of Biological Materials. One gram of sample was refluxed with 100 ml constant-boiling HCl for 20 hours. The hydrolysate was filtered through glass fiber Reeve Angel 934AH filter paper, and the sample was taken to dryness at room temperature on a rotary evaporator. The dry residue was redissolved in 1 liter of 0.1 *N* HCl. A 25.0-ml aliquot of the above sample (25 mg) was transferred into a ₹ 125-ml flat-bottom flask, lyophilized, then dried and derivatized as in Method I.

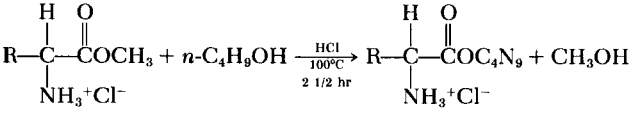
Derivatization Method—Preparation of N-trifluoroacetyl *n*-butyl Esters

General. The derivatization of amino acids present in biological samples and protein hydrolysates involves the following steps:

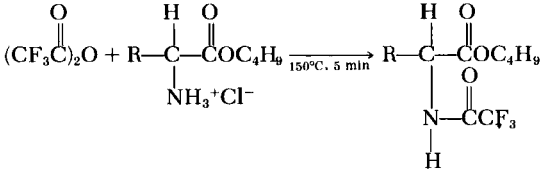
1. Removal of water to give dry amino acids.
2. Esterification of the amino acids to form methyl ester hydrochlorides:



3. Interesterification of the methyl esters to form *n*-butyl ester hydrochlorides:



4. Acylation of *n*-butyl ester hydrochlorides with trifluoroacetic anhydride to form N-trifluoroacetyl *n*-butyl esters:



Methyl ester formation is necessary to achieve solubility of cystine, histidine, and lysine in the *n*-butanol, and butyl ester formation is necessary to prevent volatilization losses and to obtain a derivative of good chromatographic separation characteristics (*8*).

Removal of Water from Protein Hydrolysates. *Method I—Vacuum evaporation.* Transfer an aqueous aliquot of the protein hydrolysate containing ca. 5 to 25 mg of total amino acids to a 125-ml ℱ 24/40 flat-bottom boiling flask containing a teflon-coated magnetic stirring bar. Place the sample flask on a Calab rotary vacuum evaporator. Immerse the flask in a water bath at 60 to 70°C, then remove the water by slowly lowering the pressure (to prevent bumping) until minimum pressure is attained.

Method II—Lyophilization of the Sample. Place the aliquot in a 125-ml ℱ 24/40 flat-bottom boiling flask as above and shell-freeze prior to being placed on an efficient lyophilizer to remove the water.

Esterification of the Amino Acids with Methanol. Add 10.0 ml of anhydrous methanol containing 1.25 meq/ml of dry HCl gas (for each 60 mg of amino acids). (If the internal standard or slope-factor methods of analysis are to be used, add at this point an exact amount of a CH_2Cl_2 solution of methyl stearate, internal standard.) Stopper the flask with a ground-glass stopper and stir the solution for 30 min at room temperature. Remove the methanol·HCl with the aid of the rotary evaporator and water-bath set at 60°C.

Interesterification of Methyl Ester Hydrochlorides. Add 10.0 ml of *n*-butanol containing 1.25 meq HCl/ml. Stopper the flask with a $CaSO_4$ drying tube and place the sample in an aluminum oil bath maintained at 100°C by a Cenco Multiple Magnetic Hot Plate Stirrer and mix the sample for $2\frac{1}{2}$ hours. Remove the *n*-butanol with the aid of the rotary evaporator at 60°C.

Sealed-Tube Acylation of *n*-butyl Ester Hydrochlorides. Add 4.00 ml of methylene chloride and 1.0 ml of trifluoroacetic anhydride. Stir for 15 min at room temperature. Transfer a 1.0-ml aliquot of the solution to a heavy-walled 12.0-ml screw-top centrifuge tube or Carius combustion tube. Tightly cap the tube with a teflon-lined cap and place the bottom of the tube in a 150°C oil bath *just to the sample liquid livel* for 5 min. *Caution:* This should be done in a hood with the oil bath behind a safety shield. Cool the tube after acylation by placing it in a beaker of water. The sample is now ready for direct injection into the gas chromatograph and analysis by GLC.

Acylation at Room Temperature. Also, acylation at room temperature of the remaining solution (4 ml) may be continued for 1 hour and 45 min (total of 2 hours). Analysis of this solution by GLC gives satisfactory results for all the amino acids except arginine and tryptophan. Comparisons of the relative peak area by both methods of acylation gives identical results.

Methods of Calculation

Method I—Relative Molar Amino Acid Composition. Calculation of the sample composition from the chromatogram involves determining the experimental peak area representing each amino acid and correcting this peak area with the response factor, $RMR_{a.a./glu}$. In this way molar percent comparisons are made possible. In our

experiments a disc integrator was used and peak areas were measured as counts.

Relative molar per cent composition:

1. Determine experimental peak area for each amino acid from chromatogram.
2. Divide each peak area by its corresponding relative molar response factor to give a corrected peak area.
3. Sum all the individual corrected peak areas.
4. Divide each corrected amino acid peak area by the sum of all the corrected peak areas.
5. Express the result as relative mole per cent.

$$\text{Relative mole \% for an amino acid} = \frac{\frac{A_{a.a.}}{RMR_{a.a./glu}}}{\sum \frac{A_{a.a.}}{RMR_{a.a./glu}} + \cdots} \times 100$$

$A_{a.a.}$ = area of amino acid peak on chromatogram

$$RMR_{a.a./glu} = \frac{\text{amino acid molar response}}{\text{glutamic acid molar response}}$$

RMR_{glu} = assigned a value of unity

The relative molar response values for the 20 natural protein amino acids relative to glutamic acid as "1" are reported in (7). These response values were obtained under normal operating conditions with a hydrogen flame-ionization detector. An investigator should confirm these reported values, e.g., lysine, aspartic acid, or an aliphatic amino acid, to establish that the analytical derivatization method is being properly followed. The relative molar response for glutamic acid is arbitrarily assigned a value of unity.

In effect, all instrumental variables are canceled when the relative mole % values are determined as described. If the relative molar response values are different from those reported (7), then the amino acid derivative is reacting with the substrate or an impurity is present with the same retention time. The values for the relative molar response for tryptophan N-ditrifluoroacetyl *n*-butyl ester and arginine N-tri-trifluoroacetyl *n*-butyl ester are 1.09 and 0.60, respectively. The assumption is made in this method of calculation that whatever changes occur in the detector response for

the glutamic acid derivative that the corresponding changes occur for the other amino acid derivatives.

Example—Method I. Relative Mole%. A known amino acid mixture (~2 to 5 mg of each) containing alanine, valine, phenylalanine, and glutamic acid was derivatized and chromatographed according to the procedure (see Table 1). The counts for the peaks and calculations are as follows.

TABLE 1

Calculation of Relative Mole % of Amino Acid—Method I

Amino acid	Counts	$RMR_{a.a./glu}$	$\frac{\text{Counts}}{RMR_{a.a./glu}}$	Mole %
Alanine	270	0.53	509	17.60[a]
Valine	340	0.68	500	17.26
Phenylalanine	418	1.12	373	12.90
Glutamic Acid	1512	1.00	1512	52.24
			2894	100.00

[a] Mole % alanine = (509 × 100)/2894 = 17.60.

Calculations—Relative Mole %. The counts for each amino acid are divided by the relative molar response for that amino acid. The RMR values for the 20 natural protein amino acids relative to glutamic acid assigned one (1) are given in (7).

Method II—Internal Standard Method. This method is used to calculate the mole %, or w/w %, for each amino acid. The calculation of the absolute amounts of amino acids in a sample is best accomplished by the use of an internal standard. The internal standard may be any compound which can be separated from the components in the sample. *n*-Butyl stearate was chosen as a suitable internal standard because it was well resolved from the amino acids on columns of the mixed phase (DEGS/EGSS-X) and 5% DC-550 on acid-washed Chromosorb W. Other fatty acid *n*-butyl esters could be employed.

The amount of an amino acid present in a sample can be calculated from information on the relationship of the molar response for the internal standard (*n*-butyl stearate) to glutamic acid when

using a flame-ionization detector. The value for this relationship may be obtained by converting 10.0 mg of glutamic acid and 5.0 mg of methyl stearate or stearic acid to their respective *n*-butyl esters, followed by acylation of the glutamic acid as described in the analytical method outlined in the section on Derivatization Method, under Experimental.

Analyze the sample by GLC, then calculate the relative molar response (RMR) ratio for glutamic acid/*n*-butyl stearate using the following formula:

$$\mathrm{RMR}_{\mathrm{glu/i.s.}} = \frac{\dfrac{\text{peak area glutamic acid}}{[\text{sample weight/molecular weight}]_{\mathrm{glu}}}}{\dfrac{\text{peak area } n\text{-butyl stearate}}{[\text{sample weight/molecular weight}]_{\mathrm{CH_3\text{-}stearate}}}}$$

Prior to esterification of the amino acids with methanol:

1. Add to the sample to be analyzed a carefully weighed amount of methyl stearate equivalent in weight to half the amount of glutamic acid or other major component present.
2. Determine the peak areas in the sample for all of the amino acids and the internal standard.
3. With information on the number of moles of internal standard added to the sample, the $\mathrm{RMR}_{\mathrm{glu/i.s.}}$ (or any other amino acid), and the sample weight of biological material, the mole %, or w/w %, of the amino acids can be calculated with the following formula:

$\mathrm{RMR}_{\mathrm{glu/i.s.}}$ = relative molar response for glutamic acid/*n*-butyl stearate

$\mathrm{RMR}_{\mathrm{a.a./glu}}$ = relative molar response for *any* amino acid to glutamic acid.

$$\mathrm{moles}_{\mathrm{a.a.}} = \frac{A_{\mathrm{a.a.}}}{\dfrac{A_{\mathrm{i.s.}}}{\mathrm{moles}_{\mathrm{i.s.}}} \times \mathrm{RMR}_{\mathrm{glu/i.s.}} \times \mathrm{RMR}_{\mathrm{a.a./glu}}}$$

Weight of amino acid = (mole wt. amino acid) × (moles amino acid)

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

The above equation for w/w or mole % of an amino acid can be developed from the relationship of the molar response of an amino acid derivative relative to an internal standard $\mathrm{RMR}_{\mathrm{a.a./i.s.}}$:

$$RMR_{a.a./i.s.} = \frac{[\text{area/mole}]\ \text{amino acid}}{[\text{area/mole}]\ \text{internal standard}}$$

$$RMR_{a.a./i.s.} = RMR_{a.a./glu} \times RMR_{glu/i.s.}$$

$$RMR_{a.a./glu} = \frac{A_{a.a.}}{[\text{sample weight/GFW}]_{a.a.}}/1.00$$

where GFW is the gram formula weight. Also,

$$RMR_{glu/i.s.} = 1.00/\frac{A_{i.s.}}{[\text{sample weight/GFW}]_{i.s.}}$$

Then,

$$RMR_{a.a./i.s.} = \frac{A_{a.a.} \times GFW_{a.a.} \times g_{i.s.}}{g_{a.a.} \times A_{i.s.} \times GFW_{i.s.}}$$

Solving for g (grams of a.a.),

$$g_{a.a.} = \frac{A_{a.a.} \times GFW_{a.a.} \times g_{i.s.}}{A_{i.s.} \times GFW_{i.s.} \times RMR_{a.a./glu} \times RMR_{glu/i.s.}}$$

Example—Method II. Internal Standard Method. Five (5 μM) of methyl stearate internal standard were added to a known amino acid mixture (~1 to 3 mg of each) containing alanine, valine, phenylalanine, and glutamic acid. A 5.000-ml aliquot of a methanol solution of the internal standard was added (1 ml containing 0.3 mg of methyl stearate).

First, make a series of independent measurements for the $RMR_{glu/i.s.}$. *n*-Butyl stearate was used as internal standard and the average response ratio was found to be 0.49. The relative molar responses ($RMR_{a.a./glu}$) for the amino acids to glutamic acid are given in (7).

The area counts for the peaks and calculations are given in Table 2.

TABLE 2

Calculation of w/w or Mole % of Amino Acid—Method II

Amino acid	Counts	$RMR_{a.a./glu}$	Moles × 10^6	Milligrams
Alanine	373	0.53	13.82	1.23
Valine	516	0.68	14.70	1.72
Phenylalanine	442	1.12	7.74	1.28
Glutamic acid	995	1.00	19.50	2.87
n-Butyl stearate	520			

Calculation of w/w %. Substitute the experimental data in the equation

$$\text{moles}_{\text{a.a.}} = \frac{A_{\text{a.a.}}}{A_{\text{i.s.}}/\text{moles}_{\text{i.s.}} \times \text{RMR}_{\text{glu/i.s.}} \times \text{RMR}_{\text{a.a./glu}}}$$

Example

$$\text{moles}_{\text{ala}} = \frac{373}{520/5 \times 10^{-6} \times 0.49 \times 0.53} = 13.8 \times 10^{-6}$$

$$\text{grams of alanine} = \text{moles}_{\text{ala}} \times \text{mole wt.}$$

$$\text{grams}_{\text{alanine}} = 13.8 \times 10^{-6} \times 89.1 = 1.23 \text{ mg}$$

Method III—Slope Factor Method. The w/w % and mole % of an amino acid in a biological sample can be calculated by this method.

$A_{\text{a.a.}}$ = area or counts of amino acid peak on experimental chromatogram (cm^2 or counts)
$S_{\text{a.a.}}$ = slope factor for amino acid (area or counts/μmole)
$A_{\text{i.s.}}$ = area or counts of internal standard peak on experimental chromatogram (cm^2 or counts)
$S_{\text{i.s.}}$ = slope factor for internal standard (area or counts/μmole)
$\mu\text{moles}_{\text{i.s.}}$ = μmoles of internal standard added to sample (μmole)
$M_{\text{a.a.}}$ = molecular weight of amino acid $\times 10^{-3}$
W = weight of protein sample (mg) hydrolyzed and derivatized

Calculation of w/w % of Amino Acid

$$= \frac{(A_{\text{a.a.}}/S_{\text{a.a.}}) \times \mu\text{moles}_{\text{i.s.}} \times (S_{\text{i.s.}}/A_{\text{i.s.}}) \times M_{\text{a.a.}}}{W \text{ of sample (mg)}} \times 100$$

Correction factor (F) compensates for injection, concentration, and instrumental variables:

$$F = \frac{[\mu\text{moles}_{\text{i.s.}}] \text{ added}}{[A_{\text{i.s.}}/S_{\text{i.s.}}] \text{ found}}$$

Then,

$$\text{w/w \% amino acid} = \frac{A_{\text{a.a.}} \times F \times M_{\text{a.a.}}}{S_{\text{a.a.}} \times W \text{ of sample (mg)}} \times 100$$

Example. See Table 3 for an example of Method III.

TABLE 3

Calculation of w/w % Amino Acid—Method III

Amino acid	Sample W, mg	$A_{a.a.}$, counts	$S_{a.a.}$,[a] counts/ μM	$A_{i.s.}$, counts	$S_{i.s.}$,[a] counts/ μM	$\mu M_{i.s.}$, added, μM	$M_{a.a.}$, mg	Found, %
Alanine	10	124	18.89	1217	72.72	16.0	0.0891	5.53
Valine	10	132	24.50	1217	72.72	16.0	0.1171	5.50
Isoleucine	10	56	29.33	1217	72.72	16.0	0.1312	2.32
Glutamic acid	10	430	35.95	1217	72.72	16.0	0.1471	17.30

[a] From Table 4.

Preparation of Calibration Curves

Calibration curves giving response versus concentration were made and the corresponding slope factors calculated. Concentration was expressed as micromoles (μM) of amino acid per 2.00 ml final volume. The solutions and calibration curves were prepared as follows: One thousand micromoles (1000 μM) of an amino acid were weighed and placed in a 200-ml glass stoppered volumetric flask. The flask was made to volume with anhydrous methanol containing 1.20 ± 0.10 meq/ml of anhydrous HCl. The solution was then esterified at room temperature for $\frac{1}{2}$ hour.

Aliquots from the stock solution were taken of 2, 5, 8, 10, 15, and 20 ml, which correspond to 10, 25, 40, 50, 75, and 100 μM, respectively. These aliquots were placed in 125-ml flat-bottom flasks with 24/40 ₮ stoppers. The excess reagents were removed from each aliquot by vacuum distillation at $65° \pm 5°$C. An exact amount, 5.000 ml, of a 50-ml solution of the internal standard, *n*-butyl stearate dissolved in CH_2Cl_2, was added to the flasks after methyl ester formation. The internal standard solution was prepared from 50 mg of *n*-butyl stearate made to 50 ml. *n*-Butanol containing 1.20 ± 0.10 meq/ml of anhydrous HCl was added to each flask in amounts equal to the above aliquots, except that 5 ml of *n*-butanol were added to the 2-ml aliquot. The solutions were then interesterified for 3 hours at $100 \pm 3°$C. After interesterification the excess reagent was removed by vacuum distillation, as described above.

The solutions were next acylated for 2 hours at room temperature using 5 ml of CH_2Cl_2 and 1.0 ml of TFAA. Arginine, histidine,

tryptophan, and cystine were acylated at 150°C for 15 min in a sealed tube as described by Stalling and Gehrke (*31*). Five-μl aliquots of the final derivatized solutions were injected directly into the gas chromatograph. The acylation method for these four amino acids was as follows: After interesterification with *n*-butanol the excess reagents were removed by vacuum distillation, then 5 ml of CH_2Cl_2 and 1 ml of TFAA were added and the solution was mixed for 5 min at room temperature. A 2-ml aliquot of this solution was taken and placed in a 15-ml centrifuge tube with a screw on plastic cap and teflon liner. One ml of additional TFAA was added. The acylation was made at 150°C for 15 min, and 5 μl of the final acylated solution after cooling were injected directly into the gas chromatograph.

TABLE 4

Slope Factors and Relative Standard Deviation for 20 Amino Acid Derivatives

Amino acid[a]	Slope factor, counts/μM	Relative standard deviation[b]
1. Alanine	18.89	1.40
2. Valine	24.50	0.27
3. Isoleucine	29.33	2.80
4. Glycine	16.26	0.19
5. Leucine	27.60	2.34
6. Threonine	22.91	0.42
7. Proline	25.86	1.66
8. Serine	19.90	0.32
9. Cysteine	18.53	1.88
10. Hydroxyproline	27.77	1.68
11. Methionine	24.62	1.94
12. Phenylalanine	41.83	3.56
13. Aspartic acid	32.39	1.33
14. Glutamic acid	35.95	1.83
15. Tyrosine	38.89	1.25
16. Lysine	31.12	1.78
17. Histidine[c]	19.08	1.99
18. Arginine[c]	21.44	1.25
19. Cystine[c]	19.81	3.27
20. Tryptophan[c]	39.12	0.47
21. Butyl stearate	72.72	1.50

[a] Separated on 0.75/0.25 w/w % DEGS/EGSS-X coated on 60/80 mesh a.w. Chromosorb W.

[b] $\sigma\% = (S_b/b) \times 100$; $S_b = [\Sigma(y - \bar{y})^2 - b\Sigma(x - \bar{x})(y - \bar{y})/(n - 2)\Sigma(x - \bar{x})^2]^{1/2}$; S_b = standard deviation of the slope b at mean $\bar{x}$; y = area at x μmoles.

[c] Separated on 5% w/w DC-550 coated on a.w. 60/80 mesh Chromosorb W.

The calibration curve for each amino acid was prepared from three independent observations at each of the six different concentrations. The slope of each curve (Table 4) was calculated by the least squares approximation of the regression curve, Fig. 2. The calibration curve for *n*-butyl stearate was prepared using glutamic acid as the internal standard in equal micromolar amounts. Five μl of the final solution (1 mg/ml) of *n*-butyl stearate in chloroform was chromatographed independently 10 times, to obtain a good statistical average.

As a chromatographically pure sample of cysteine was difficult to obtain, it was necessary to determine by gas-chromatographic analysis the amount of proline (6%) in the cysteine and to make the necessary corrections in the calibration curve for cysteine.

Analysis of Amino Acid Mixtures

Various known mixture combinations of the amino acids were prepared by weighing 50 or 100 mg of each amino acid and placing this mixture into a 250-ml volumetric flask (Tables 5, 6, 7, and 8). The flask was made to volume with 0.1 *N* HCl. Then, 20- or 25-ml aliquots of each solution were placed in 125-ml ₹ 24/40 flasks. The sample solution was evaporated to dryness at 60°C by vacuum distillation on a Calab rotary evaporator or lyophilized at room

TABLE 5

Gas-Liquid Chromatographic Analysis of Amino Acids in a Mixture

	Milligrams of amino acid					
		Recovered by GLC[a]				Ion exchange[b]
	Added	1	2	3	Av.	av.
Valine	100	105.4	100.9	103.9	103.4	102.8
Glycine	100	101.7	98.5	97.9	99.4	100.1
Threonine	100	98.3	103.6	98.8	100.2	97.2
Aspartic acid	100	99.8	101.4	101.8	101.0	101.5
Lysine	100	99.0	100.4	101.8	100.4	104.1

[a] Mixture separated on a 1 m × 3 mm I.D. 0.75/0.25 w/w % DEGS/EGSS-X mixed phase coated on 60–80 mesh a.w. Chromosorb W column; 5 μl of 6 ml final solution injected.

[b] Average of at least two determinations.

temperature for 18 hours at less than 200 μ of pressure. Also, some of the samples were then completely dried over P_2O_5 in a vacuum desiccator for 24 hours at room temperature. After drying, the mixtures were derivatized by the method described in the Experimental Method section.

TABLE 6

Gas-Liquid Chromatographic Analysis of Amino Acids in a Mixture

Amino acid	Milligrams of amino acid					Ion exchange[b]
	Added	Recovered by GLC[a]			Av.	av.
Isoleucine	50.0	48.6	50.2	49.7	49.5	49.6
Leucine	50.0	50.9	50.3	49.2	50.1	49.6
Hydroxyproline	50.0	50.3	50.9	50.7	50.6	50.0
Aspartic acid	50.0	49.9	50.7	50.9	50.5	50.7
Phenylalanine	50.0	49.5	51.9	49.9	50.1	50.2
Glutamic acid	50.0	49.7	49.3	50.4	49.8	49.9
Lysine[c]	50.0	48.7	49.3	50.1	49.4	52.0
Arginine[c]	50.0	50.9	50.1	51.4	50.8	50.1
Cystine[c]	50.0	51.3	50.7	51.8	51.3	50.4

[a] *n*-Butyl stearate internal standard, 5 μl of 6 ml final solution injected directly on column. Column 1 m × 3 mm of 0.75/0.25 w/w % DEGS/EGSS-X on 60–80 mesh a.w. Chromosorb W.

[b] Average of at least two determinations.

[c] Column 1 m × 3 mm of 5 w/w % DC-550 on 60/80 mesh a.w. Chromosorb W.

TABLE 7

Gas-Liquid Chromatographic Analysis of Amino Acids in a Mixture

Amino acid	Milligrams of amino acid			
	Added	Recovered by GLC[a,b]		Av.
Methionine	50.0	49.3	50.1	49.7
Tyrosine	50.0	48.9	50.1	49.5
Histidine	50.0	49.1	49.9	49.5
Arginine	50.0	51.2	50.5	50.9
Cystine	50.0	50.0	50.3	50.2

[a] *n*-Butyl stearate internal standard, 5 μl of 6 ml solution injected directly on column.

[b] Lyophilized samples dried for 24 hours over P_2O_5 at room temperature in vacuum. Column 1 m × 3 mm of 5 w/w % DC-550 on 60/80 mesh a.w. Chromosorb W.

TABLE 8

Gas-Liquid Chromatographic Analysis of Amino Acids in a Mixture

Amino acid	Milligrams of amino acid			
	Added	Recovered by GLC[a]		Av.
Glycine	50.0	49.7	50.5	50.1
Valine	50.0	49.9	50.8	50.4
Threonine	50.0	49.8	50.3	50.1
Phenylalanine	50.0	51.0	50.2	50.6
Glutamic acid	50.0	49.7	50.3	50.0
Lysine	50.0	50.1	49.3	49.7

[a] *n*-Butyl stearate internal standard, 5 μl of 6 ml final solution injected directly on column. Column 1 m × 3 mm of 0.75/0.25 w/w % DEGS/EGSS-X on 60–80 mesh a.w. Chromosorb W.

Determination of Yields of Amino Acid Derivatives

The following procedure developed by Lamkin and Gehrke (*18*) was employed to determine the per cent conversion to derivative of the amino acids carried through the analytical method.

First, pure reference standard N-trifluoroacetyl *n*-butyl ester derivatives of all the amino acids were prepared and purified by high-vacuum sublimation (*17,30*). Then, independent 10.0-mg samples of each amino acid were weighed into 125-ml ℱ 24/40 flasks, and 10.0 ml of anhydrous methanol containing 1.25 ± 0.05 meq/ml of anhydrous HCl were added. The resulting solution was stirred with a magnetic stirrer at room temperature for 30 min. The methanol was removed by vacuum evaporation at 60°C, then 10.0 ml of *n*-butanol containing 1.25 ± 0.05 meq/ml of anhydrous HCl were added to the flask. The flask was fitted with a $CaSO_4$ drying tube and placed in a 90 ± 1°C oil bath and stirred for 180 min. The *n*-butanol was removed by vacuum evaporation at 60°C, and 5.00 ml of methylene chloride and 0.50 ml of trifluoroacetic anhydride were added. The flask was then stoppered and stirred at room temperature for 120 min with a magnetic stirrer. The solvents were removed at room temperature and 2.000 ml of a 10.000-ml solution of anhydrous methylene chloride containing an internal standard was added to each of the sample flasks and to a flask containing a weighed amount of the purified derivative of the amino acid (ca. 15 to 25 mg). The internal standard was the N-trifluoroacetyl

n-butyl ester derivative of glutamic acid, aspartic acid, or phenylalanine prepared from 50.0 mg of amino acid also carried through the above procedure. Arginine and tryptophan were acylated in a sealed tube at 150°C for 5 min (*31*). Five μl of each solution were chromatographed.

The recovery experiments are based on the fact that *equal amounts* of internal standard (*I*) are added to the *sample* and *pure reference* amino acid flasks. The yield was calculated by the formula given below.

The yield was determined by comparing the detector response from the *analytical samples* taken through the method with that for the *reference standard* derivatives. The yield was expressed as a per cent. Conversion of amino acids to their respective derivatives was found to be quantitative (*7*). The data are the subject of another paper.

Percent Conversion of Amino Acid to Derivative

$$\% \text{ yield} = \frac{A_a/A_I \text{ with } a}{A_R/A_I \text{ with } R} \times \frac{W_{R \text{ used}}}{W_{Rc}} \qquad (1)$$

$W_{Rc} \cong W_a$ *used* is calc. from

$$W_a/M_a = W_{Rc}/M_R$$

$W_{Rc} = W_a \times M_R/M_a$; then substitute in (1)

$$\% \text{ yield} = \frac{A_a \times A_{IR} \times W_R \times M_a}{A_{Ia} \times A_R \times W_a \times M_R} \times 10^2$$

A_a = peak area for amino acid derivative in analytical method (ca. 10 mg)

A_{Ia} = peak area for internal standard in amino acid *sample* flask

A_R = peak area for weighed amount of *pure reference* amino acid

A_{IR} = peak area for internal standard in *pure reference* amino acid flask

W_a = weight of amino acid used

M_a = mole weight of amino acid

W_R = weight of *pure reference* amino acid used

M_R = mole weight of the pure N-trifluoroacetyl *n*-butyl ester derivative of the *pure reference* amino acid

RESULTS AND DISCUSSION

Chromatographic and Substrate Considerations

Complete resolution of a mixture of 20 protein amino acids by GLC was reported by Gehrke and Shahrokhi (8) using a mixed phase column of 0.75/0.25 w/w % of DEGS and EGSS-X. This column gave good resolution of isoleucine, glycine, and leucine, and threonine and proline. However, some difficulty has been observed in maintaining the complete separation of isoleucine and glycine, especially if the column is operated at its maximum temperature limit for an extended period of time. Also, prolonged conditioning at temperatures greater than 180°C causes the resolution to be impaired. Our experiments show that EGSS-X undergoes some kind of a modification at high temperatures. This is responsible for the change in separation characteristics of the DEGS/EGSS-X mixed phase column.

Investigations on a number of liquid phases of different composition are in progress to find a single liquid substrate that will resolve the N-trifluoroacetyl *n*-butyl ester derivatives of all 20 natural protein amino acids. It is clear from our current research that a liquid phase with separation characteristics intermediate between DEGS and a carbowax is required. However, studies with support-coated open tubular columns (33) containing the substrate Co-880 (phenoxyethanol polymer) caused the loss of threonine. Earlier studies with packed carbowax columns also resulted in the loss of cystine due to a derivative column interaction. The liquid phase employed should be thermally stable to at least 240°C.

A study of the separation characteristics of commercially available polyesters having increasing acid chain lengths led to the observation that their separation ability improved as the chain length of the acid increased. Acids, having chain lengths of C_4, C_6, and C_{10}, esterified with neopentyl glycol or butane-1,4-diol were examined as liquid phases. Neopentyl glycol sebacate (C_{10}) was shown to be the best of all the liquid phases investigated. The separation of 19 of the amino acids and an internal standard, *n*-butyl stearate, is shown in Fig. 1. Excellent resolution in 62 min was achieved with a *single* substrate column. Continuing evaluation of even longer chain polyesters is currently underway.

Stalling et al. (33) have studied support-coated open tubular

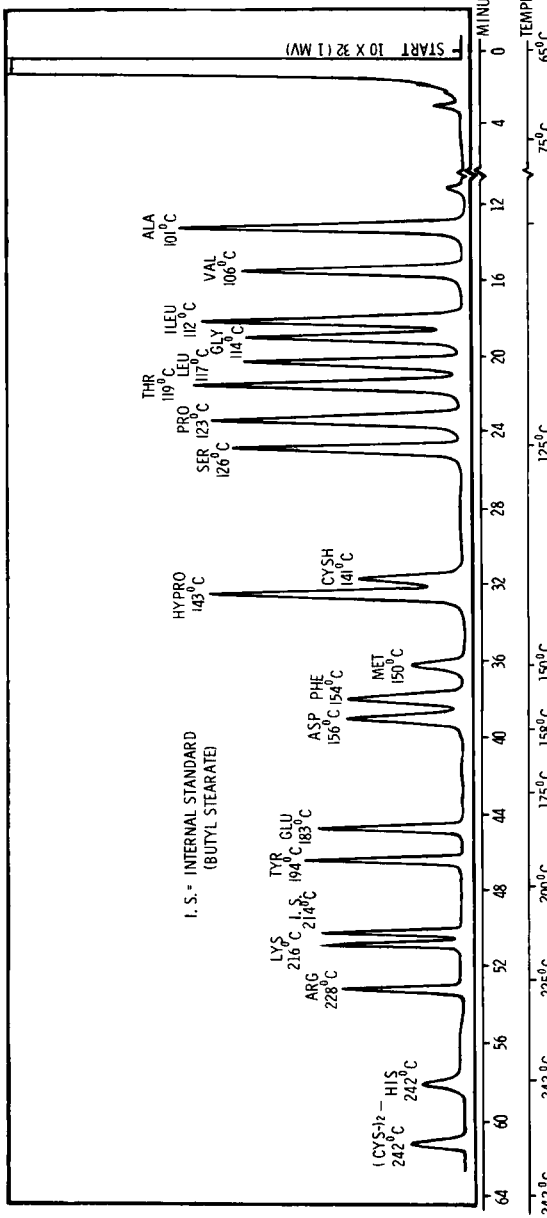


FIG. 1. Separation of amino acid derivatives with neopentyl glycol sebacate. Each peak represents 5 μg amino acids. Column: 1.5 m × 3.7 mm I.D. of 0.5 w/w % neopentyl glycol sebacate coated on 80/100 a.w. Chromosorb G. Microtek Model 220 gas chromatograph with flame-ionization detectors; 1-mv potentiometric recorder. Carrier gas flow: 64 ml/min N_2.

columns at low loadings and they appear to offer advantages over normal packed columns in that they are much more efficient. With the SCOT columns, separation of the natural protein amino acids may be more readily achieved by a *single* liquid phase than with the normal packed columns. Investigations are now in progress on the evaluation of neopentyl glycol sebacate SCOT columns.

Calibration Curves

The data from the calibration curves for the 20 natural protein amino acids over a 0-to-100 μM concentration range were statistically examined and typical curves are shown in Fig. 2. The statistical bias of the calibration curves at the 95% confidence level was not significant. No deviations from linearity were observed. The slope factors (area/μM) as determined with a flame-ionization detector and relative standard deviations (RSD) of the slope at the mean value for the protein amino acid N-trifluoroacetyl *n*-butyl ester derivatives are given in Table 4. Except for phenylalanine and cystine the RSD values are less than 3%. This per cent devia-

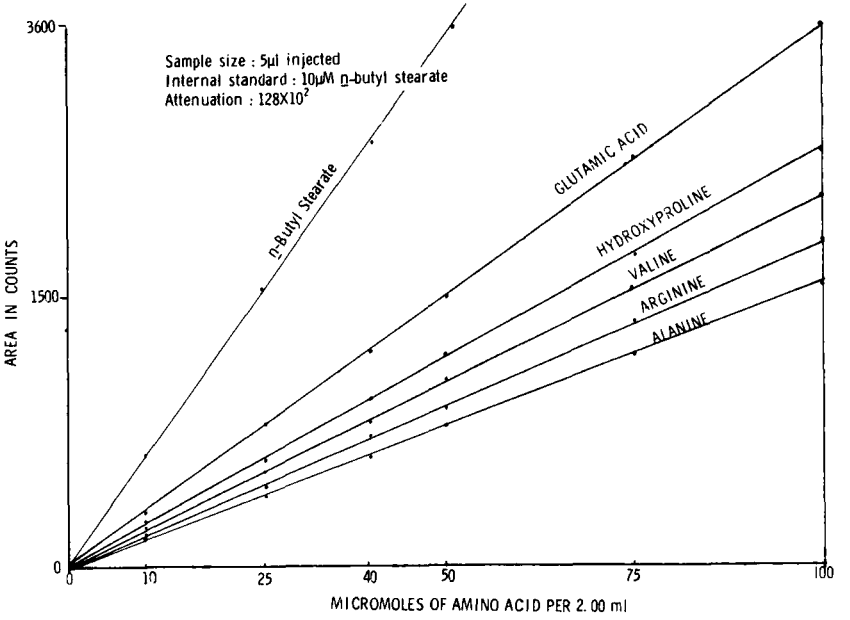


FIG. 2. Typical calibration curves.

tion is of approximately the same magnitude as the error involved in measuring the peak area with a disc integrator; thus, the precision and accuracy of amino acid analyses by gas-liquid chromatography is excellent.

Gas-Chromatographic Analysis of Synthetic Amino Acid Mixtures and Protein Hydrolysates

Comparison of data from the analyses of known synthetic mixtures of amino acids by gas-liquid and ion-exchange chromatographic methods afforded a convenient means of determining the accuracy and precision of the two methods. A five-component mixture of representative amino acids was analyzed by both techniques. The results are given in Table 5 and showed agreement within the same error for both methods. Additional information on the precision and accuracy of the gas-chromatographic method

TABLE 9

Relative Standard Deviation for Gas-Chromatographic Analysis of Amino Acid Mixtures

Amino acid	Relative standard deviation[a]
Alanine	1.71
Valine	2.28
Isoleucine	1.64
Glycine	2.04
Leucine	1.84
Threonine	0.86
Proline	1.91
Serine	0.81
Hydroxyproline	0.66
Methionine	1.12
Phenylalanine	1.56
Aspartic acid	1.06
Glutamic acid	1.40
Tyrosine	1.16
Lysine	1.42
Histidine	1.28
Arginine	1.36
Cystine	1.12

[a] Computed from at least three independent determinations.

obtained from the analysis of other synthetic mixtures is shown in Tables 6, 7, and 8. The results of these analyses were in excellent agreement with the known values. The relative standard deviations calculated from these data are given in Table 9. All the relative standard deviations were in the range 0.66 to 2.28%.

The developed gas-chromatographic method was applied to biological samples and compared with ion-exchange data on the same samples. Data from the comparative analyses of the acid hydrolysates of three proteins, bovine serum albumin, kappa casein, and a commercial soybean protein preparation (Ralston-Purina Edi-Pro) are given in Tables 10, 11, and 12. Of special im-

TABLE 10

Amino Acid Analysis of Bovine Serum Albumin[a]

Amino acid	w/w %				
	Gas chromatography[b]			Av.	Ion exchange[c]
Alanine	5.71	5.53	5.67	5.67	5.64
Valine	5.55	5.69	5.50	5.58	5.44
Isoleucine	2.29	2.32	2.22	2.28	2.38
Glycine	1.63	1.53	1.66	1.61	1.70
Leucine	11.61	12.26	11.85	11.90	11.68
Threonine	5.43	5.31	5.30	5.34	5.46
Proline	4.79	4.71	4.72	4.74	4.78
Serine	3.81	4.11	3.65	3.86	4.05
Methionine	0.71	0.68	0.59	0.66	0.70
Phenylalanine	6.47	6.50	6.37	6.44	6.42
Aspartic acid	10.39	10.36	10.19	10.31	10.27
Glutamic acid	17.04	16.75	16.70	16.83	16.43
Tyrosine	5.21	4.97	5.33	5.17	5.24
Lysine	12.31	12.21	12.03	12.18	12.16
Arginine	5.55	5.11	5.40	5.35	5.69
Histidine	3.38	3.44	3.30	3.38	3.64
Cystine	5.37	5.45	5.40	5.41	5.65
				106.71	107.33

[a] Protein hydrolyzed for 18 hours at 105°C in a sealed tube with constant-boiling HCl.

[b] *n*-Butyl stearate used as internal standard.

[c] Each value represents an average of two independent determinations, norleucine as internal standard.

portance is the total w/w % of amino acids found by both methods for the three proteins, as the agreement was within 1% absolute. Excellent agreement of the quantities of individual amino acids found by both methods was also obtained. A chromatogram of the bovine serum albumin hydrolysate is shown in Fig. 3, representing 25 μg of total amino acid injected. A combination of temperature program rates was employed to facilitate the separation of the N-trifluoroacetyl *n*-butyl ester derivatives.

The data from the gas-chromatographic analyses of synthetic mixtures of amino acids and protein hydrolysates is in excellent agreement with ion-exchange analyses. This conclusively demonstrates that quantitative analysis of amino acids by gas chromatography can be achieved rapidly and accurately.

TABLE 11

Amino Acid Analysis of Kappa Casein[a]

Amino acid	w/w % GLC[b]	w/w % Ion exchange[b]
Aspartic acid	7.75	7.88
Threonine	6.20	6.34
Serine	5.54	5.49
Glutamic acid	17.92	18.65
Proline	8.82	9.25
Glycine	0.87	0.94
Alanine	5.45	5.51
Valine	5.14	5.19
Cystine	0.42	0.34
Methionine	0.30	0.43
Isoleucine	6.27	6.31
Leucine	5.07	5.14
Tyrosine	6.97	6.81
Phenylalanine	2.96	3.07
Ammonia		2.42
Lysine	6.31	6.14
Histidine		2.04
Arginine	3.55	3.79
	89.95	88.86

[a] Protein hydrolyzed for 18 hours at 105°C in a sealed tube with constant-boiling HCl.

[b] Each value represents a single determination.

TABLE 12

Amino Acid Analysis of a Soybean Protein[a]

Amino acid	w/w %			
	Gas chromatography[b]		Av.	Ion exchange[c]
Alanine	3.31	3.48	3.39	3.53
Valine	4.15	4.36	4.26	4.23
Isoleucine	4.08	4.21	4.15	4.24
Glycine	3.23	3.47	3.35	3.57
Leucine	6.92	6.98	6.95	6.80
Threonine	2.34	2.71	2.53	2.98
Proline	4.43	4.53	4.48	4.44
Serine	4.17	4.23	4.20	4.48
Methionine	0.82	0.71	0.77	0.84
Phenylalanine	4.35	4.40	4.38	4.51
Aspartic acid	10.85	10.97	10.91	11.08
Glutamic acid	18.93	19.03	18.98	18.73
Lysine	5.30	5.39	5.35	5.24
Tyrosine	3.50	3.37	3.44	3.21
Arginine	6.13	6.32	6.23	6.36
Cystine	0.56	0.44	0.50	0.50
			83.67	84.74

[a] Protein hydrolyzed for 18 hours at 105°C in a sealed tube with constant-boiling HCl.

[b] *n*-Butyl stearate used as internal standard.

[c] Each value represents an average of four independent determinations, norleucine as internal standard.

Comments on Methods of Calculation

Method I—Relative Mole %. This method for the calculation of the relative molar amino acid composition of a biological sample is:

1. Experimentally the simplest.
2. Useful for comparative purposes. One can follow purity of a preparation, cross-check the information with that from Stein-Moore, or analyze many samples for amino acid ratios, i.e., in genetic studies in plant breeding to determine ratios of lys/leu/met, etc.
3. Does not give quantitative data on an *absolute* basis.

Method II—Internal Standard Method. This internal standard method is useful for the determination of the *absolute* amount of an amino acid in a sample.

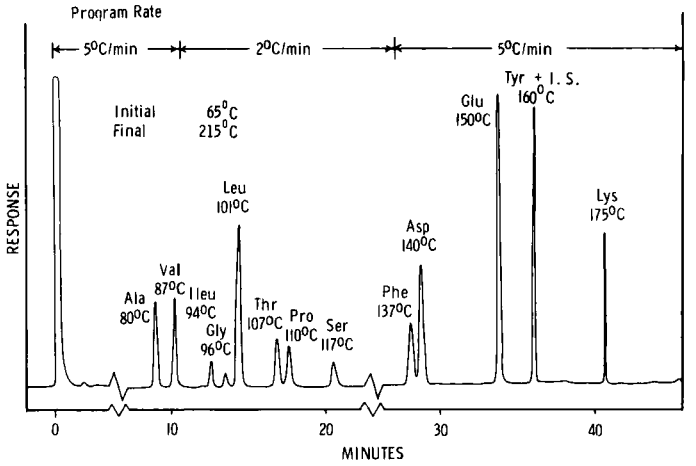


FIG. 3. Chromatogram of bovine serum albumin hydrolysate. Sample size: 5 μl (25 μg total amino acids). Column: 1.00 m × 4 mm I.D. borosilicate glass packed with 60/80 mesh a.w. Chromosorb W and *mixed* substrate phase of 0.75/0.25 w/w % of DEGS/EGSS-X.

1. Gives exact mole % or w/w % of an amino acid with a minimum of instrumental calibration.
2. Useful when quantitative data for a few amino acids are required.
3. Does not require the preparation of calibration curves for the amino acids. This is difficult if the pure reference standards are not available.
4. Does not require any calibration curve for the internal standard.
5. This method does require information on the *exact* amount of internal standard added to the sample flask.
6. Does require information on the relationship of $RMR_{glu/i.s.}$ (or any other amino acid) and the $RMR_{a.a./glu}$.
7. Dilution or concentration of sample, after addition of internal standard are of little importance.
8. Amount of derivatized sample injected is not critical as long as a good size peak is obtained.

Method III—Slope Factor Method. This method for the calculation of mole % or w/w % of an amino acid is the most elaborate and extensive; however, this method is most applicable in the interpre-

tation of the analytical data for protein hydrolysates or other complex biological samples.

1. Good calibration curves must be prepared for each amino acid over the required concentration range.
2. Requires a complete calibration curve for the internal standard over the required concentration range.
3. This method allows a better statistical evaluation of the analysis as more observations are employed in the preparation of the calibration curves.
4. Allows a confidence interval to be established for any point on the calibration curve.
5. The *exact* amount of internal standard added to the sample flask must be known.
6. Dilution or concentration of sample after addition of internal standard are of little importance.
7. Amount of derivatized sample injected is not important as long as a good-size peak is obtained.

Quantitative and Qualitative Concentration Ranges

For quantitative analysis a good workable concentration range is injection of 1 to 35 μg of each amino acid. It is best to work at a level of about 50 μM ($\sim$5 mg) of amino acid per 2 ml, and inject 5 μl ($\sim$12 μg of amino acid). The above weights of amino acids are obtained if aliquots of a hydrolysate containing 10 to 30 mg of protein are taken and the final volume of the sample solution is made to about 1 ml.

If necessary, a lower workable concentration range is 50 to 500 ng for each amino acid. This range can easily be reduced to 10 ng and still retain reproducible peaks of measurable area. As given on the calibration curves, one normally works at higher concentrations. The calibration curves (Fig. 2) were prepared over the range 10 to 100 μM of amino acid per 2.00 ml final volume, and 5μl of this solution were injected into the chromatograph.

The minimum detectable limits (MDL) for *qualitative* work are 0.1 to 1 ng for each amino acid injected. The amino acids with lower retention temperature have a lower MDL than those with higher retention temperature. This is due to substrate bleeding at the higher temperatures with a consequent noise increase.

The use of an alkali-metal nitrogen flame detector as reported

by Aue et al. (*1*) increases the response about two orders of magnitude for the amino acids and allows for effective discrimination against nonnitrogen compounds.

Gas Chromatography of Nonprotein Amino Acids

Data are presented in Table 13 on the relative molar response, $RMR_{a.a./glu}$, and retention temperature for the N-trifluoroacetyl-*n*-butyl ester derivative of 15 nonprotein amino acids. The samples were acylated at two different sets of experimental conditions (25°C for 2 hours, and 150°C for 5 min). The results were in excellent agreement, indicating that the derivatization reaction was reproducible and quantitative at either acylation condition. Good peak

TABLE 13

Relative Molar Response and Retention Temperature of Nonprotein Amino Acids

Amino acid	Relative molar response,[a] acylation method		Retention[b] temp., °C
	25°C; 2 hr	150°C; 5 min	
Ortho tyrosine	0.97	0.99	169
Meta tyrosine	0.94	0.96	175
3,4-Dihydroxy phenylalanine	0.97	0.97	176
Paraamino Benzoic acid	1.03	1.03	144
β-Amino isobutyric acid	0.59	0.59	114
γ-Amino butyric acid	0.66	0.65	140
ε-Amino caproic acid	0.88	0.89	165
α-Amino *n*-butyric acid	0.65	0.66	125
β-Alanine	0.55	0.55	106
Norleucine	0.82	0.83	112
Norvaline	0.75	0.75	106
Dimethyl glycine	0.67	0.67	116
Ornithine	0.92	0.93	174
Ethionine	0.64	0.65	162
Asparagine[c]	0.89	0.89	174

[a] Relative molar response = $[\text{area/mole}]_{\text{amino acid}}/[\text{area/mole}]_{\text{glutamic acid}}$.

[b] Retention temperature on 1 m × 3 mm I.D. Column of 5% w/w DC-550 coated on 60/80 mesh a.w. Chromosorb W; initial temperature, 80°C; program rate 5°C/min; final temperature 225°C; 45 ml/min N_2 carrier gas.

[c] Relative molar response and retention temperature identical to aspartic acid.

symmetry was also obtained. In another experiment (*34*), reproducible chromatographic peaks were obtained for the N-O-trimethylsilyl trimethylsilyl esters of tyrosine, mono- and diiodotyrosine, triiodothyronine, and thyroxine.

Notes on the Gas-Chromatographic Method

1. *Vacuum evaporator.* The use of vacuum rotary evaporators, in which methanol or *n*-butanol HCl comes in direct contact with metal, stainless steel, or Monel, is to be strictly avoided, as the chromium and other metals in these alloys form salts with the amino acids. This prevents the formation of the volatile derivatives. The use of an all-glass, or preferably all-teflon, evaporator is therefore recommended.

2. *Closed-tube acylation.* There are dangers involved in using a glass system under pressure at an elevated temperature, and adequate precautions should be taken to prevent possible injury from fragments of glass or hot oil in the event of a sealed-tube failure. The use of a wraparound shield such as supplied by the I²R Company gives adequate protection. The acylation should also be conducted in a well-ventilated hood.

3. *Cleaning of syringes.* Immediate cleaning after use of the Hamilton syringes by consecutive washings with water, acetone, and methylene chloride using an aspirator will prolong their useful life and minimize the possibility of cross-sample contamination.

4. *Removal of vapor bubbles in syringe.* Vapor bubbles arising from the highly volatile methylene chloride and trifluoroacetic anhydride can be removed by inserting the end of the syringe needle into a silicone rubber septum and carefully pushing down on the plunger to condense the vapors. The use of a model 701-N syringe guide helps to prevent bending the plunger and aids in holding the syringe. Placing one's fingers on the syringe barrel may cause the solvent to vaporize in the barrel and result in errors in injection volume.

5. *Anhydrous storage of acylated samples.* The acylated samples may be stored under anhydrous conditions in small screw-capped vials (2 to 10 ml) which have teflon liners in the caps. Cleaning and drying the vials at 100°C in an oven prior to use is recommended. The clean, dried vials can then be stored in a desiccator until needed. The derivatives of arginine, histidine, tyrosine,

threonine, and serine are prone to hydrolysis unless maintained free of moisture from the air.

Removal of the solvents after acylation is not recommended, as it is difficult to prevent the samples from coming into contact with moist air and subsequent hydrolysis. The peaks for CH_2Cl_2, TFAA, and TFA come over very early in the chromatogram and do not interfere with the amino acid peaks.

6. *100°C interesterification.* It is necessary to interesterify the amino acids at 100°C for 150 min to obtain quantitative results for cystine, threonine, histidine, and lysine. This is mainly due to solubility problems.

7. *Chromatographic supports.* Silanized supports have not been found satisfactory, in that peak shape was degraded as well as a loss in column efficiency. In general, acid-washed supports are better than non-acid-washed supports. Acid-washed Chromosorb G, 80–100 mesh at liquid phase loadings one half that for Chromosorb W has been found superior to any support tested thus far. Column lengths greater than 1.5 to 2.0 m make elution of cystine, histidine, and arginine difficult.

8. *On-column or glass-lined injection.* Direct on-column or glass-lined injection ports have eliminated decomposition of threonine and arginine observed when all-metal flash heaters were employed. When very clean (new) metal injectors were employed at temperatures of less than 250°C, no decomposition was observed; however, when these injectors were used after samples containing nonvolatile materials were injected, some decomposition was observed. This indicates that deposits in the injector could be responsible for the observed decomposition. The use of glass-lined injection ports which can be removed and cleaned when deposits form is a satisfactory solution to this problem.

9. *Purity of nitrogen carrier gas.* The use of 99.998% purity nitrogen gas as carrier gas resulted in improved baseline bleed-off after conditioning of the columns was completed. Also problems associated with the elution of cystine were eliminated.

10. *Removal of water from biological samples.* Water can be removed from aliquots of protein hydrolysates by four methods. Excess water is first removed from the sample by lyophilization or rotary vacuum evaporation. The sample is then dried as follows:

a. Desiccant drying. Over P_2O_5 for 24 hours at room temperature and under vacuum.

b. Desiccant drying. Over P_2O_5 for 6 hours at 60 ± 5°C and under vacuum.

c. Azeotropic distillation. Add 10 ml CH_2Cl_2 and remove by vacuum evaporation at 65 ± 5°C, then repeat.

d. Chemical drying. Add 2.0 ml of 2,2-dimethoxypropane to 10 ml of methanol·HCl reagent. Remove solvents by vacuum evaporation at 60°C, then repeat.

11. *Application to biological samples.* To apply the gas-chromatographic method to biological samples, compounds which give rise to interferring peaks must be eliminated. In the analysis of samples containing fatty acids or lipids, exhaustive extraction with diethyl ether was satisfactory to eliminate fatty acid interference, i.e., soybean meal. Removal of volatile fatty acids can be achieved by vacuum evaporation at 60°C. The removal of amines and sugars is probably best accomplished with a short ion-exchange column cleanup. The choice of the proper method of eliminating interferring compounds in biological samples is best made by individual consideration of each sample type.

12. The separation of *n*-butyl stearate and the derivative of tyrosine has not been achieved with different lots of DEGS used in making the mixed-phase column of DEGS and EGSS-X. This is not a problem with columns of neopentyl glycol sebacate.

13. A sealed tube is synonymous with a closed tube.

14. The developed gas-chromatographic method of analysis offers the following: less initial instrument cost, more instrument versatility, more rapid analysis, and greater sensitivity.

15. Quantitative reference standard solutions of N-trifluoroacetyl *n*-butyl amino acid esters available from Regis Chemical Co., 1101 N. Franklin St., Chicago, Ill. 60610.

SUMMARY AND CONCLUSIONS

A quantitative gas-chromatographic method has been developed and applied to the analysis of the 20 protein amino acids and other nonprotein amino acids. A procedure is described for the conversion of amino acids to their N-trifluoroacetyl *n*-butyl esters. This procedure gives single reproducible derivatives.

Pure derivatives as reference standards were prepared and employed to determine the per cent conversion of the amino acids in the analytical procedure. The yields were excellent (98 ± 3%) for

all the amino acids when the interesterification was conducted at 100°C for 150 min. Tryptophan and arginine when acylated in a closed tube at 150°C for 5 min gave 95 and 99% conversion, respectively, to single derivatives with suitable gas-chromatographic properties. Room-temperature acylation of the arginine *n*-butyl ester hydrochloride yields a trifluoroacetate salt not suitable for gas chromatography. Closed-tube acylation studies of mixtures of amino acids at 150°C showed no interactions or adverse side reactions.

Calibration curves were prepared for the 20 protein amino acids. They were linear and showed no statistical bias. The relative standard deviations for the curves were in the range 1 to 3.5%. The concentration range employed in routine analysis of protein hydrolysates was 10 to 100 μM per 2-ml total volume, and 5 μl of this solution were injected into the gas chromatograph. An internal standard, *n*-butyl stearate, was employed to place all calculations on an absolute concentration basis.

Three methods of calculating concentrations of amino acids in protein hydrolysates or biological materials from the gas-chromatographic peak areas are described. The merits and applicability of calculations using the mole %, internal standard, or slope factor calculation methods are discussed. Example problems are included.

Comparisons of the results obtained from the analysis of representative mixtures of amino acids by gas chromatography gave excellent agreement with theory and ion exchange. Analysis of the acid hydrolysates of bovine serum albumin, kappa casein, and a commercial soybean protein gave equivalent results within 1% absolute of the total amount of amino acids obtained by ion-exchange analysis. The method was also applied to additional nonprotein amino acids, and the relative molar response and retention temperatures are given.

For good quantitative analysis of amino acids by gas chromatography, the concentration range (amount of amino acid injected) is 0.05 to 35 μg. For qualitative analysis the concentration range is 0.1 to 1.0 ng injected.

The data from gas-chromatographic analyses of synthetic mixtures of amino acids and protein hydrolysates has been shown to be in excellent agreement (within 1% absolute for total amino acids present) with ion-exchange chromatographic analyses. This demon-

strates that quantitative analysis of amino acids by gas chromatography can be achieved rapidly and accurately.

Of significant advantage is that a simple GLC method for the identification and estimation of the amino acid composition of protein hydrolysates can be carried out without purchase of expensive equipment of limited single-purpose application. Moreover, with the highly sensitive detectors now available, it is possible to identify and measure smaller amounts of amino acids than has heretofore been possible with other techniques. This gas-chromatographic method for the analysis of amino acids has broad implications in many areas of biological importance. This procedure will strongly support and advance studies in genetics, protein biochemistry, agricultural sciences, medical research, and nutrition, as well as in space-related research.

Acknowledgments

The authors express thanks and appreciation to George Gille and Frank Shahrokhi, graduate students, Department of Agricultural Chemistry, for their assistance in the development and application of the gas-chromatographic method. Data on analyses of amino acid mixtures and protein hydrolysates represents work to be included in the M.Sc. thesis of Mr. Gille. Special thanks are extended to James P. Ussary, Instructor in Agricultural Chemistry, for supervisory and technical advice on the ion-exchange analysis of the synthetic amino acid mixtures and protein hydrolysates. We also wish to acknowledge Robert Zumwalt, who assisted in the gas-chromatographic separation studies.

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Received by editor October 28, 1966
Submitted for publication November 18, 1966