CHROM. 4992

GAS–LIQUID CHROMATOGRAPHY OF AMINO ACIDS IN BIOLOGICAL SUBSTANCES*

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(Received August 12th, 1970)

SUMMARY

The development of a gas–liquid chromatographic (GLC) method for the quantitative analysis of amino acids in complex biological substances, specifically blood plasma and urine, has been achieved. The amino acids present in these physiological fluids were quantitatively isolated by ion-exchange methods and retained on the ion-exchange resin while the substances which interfere with the GLC analysis passed through the column and were discarded. The amino acids were then eluted from the column, derivatized to their N-trifluoroacetyl (N-TFA) *n*-butyl esters and analyzed by GLC. Quantitative recovery of the amino acids from the cation and anion-exchange clean-up columns, and amino acids in blood and urine were successfully carried out.

Also, techniques for the analysis of amino acids over a wide range of concentrations were developed. Analyses were made on aliquots of human blood plasma containing only 200 μg of total amino acids, and the results obtained at this level of concentration were both accurate and precise. Further, quantitative data were obtained with samples containing only 20 μg of total amino acids, and semiquantitative analyses were performed on samples containing 2 μg of total amino acids. The data obtained by the GLC method were in excellent agreement with results by classical ion exchange.

Investigations on acylation of the amino acid *n*-butyl esters have shown that the optimum molar ratio of TFAA/amino acids is 50:1 with regard to reproducibility of acylation, stability of the derivative, and maintenance of a small sample volume (> 75 μl).

Experiments involving concentration of the derivatized samples have shown

* Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 6056. Approved by the Director. Supported in part by grants from the National Aeronautics and Space Administration (NGR 26-004-011), the National Science Foundation (GB 7182) and the United States Department of Agriculture (12-14-100-8468 (34)).

** Experimental data taken in part from doctoral research, University of Missouri, NSF predoctoral research fellow.

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that the N-TFA *n*-butyl esters have a significant advantage over the N-TFA methyl esters in that evaporative losses do not occur on concentration of the sample at room temperature; whereas serious losses result when the N-TFA methyl esters of the amino acids are concentrated to a smaller volume.

INTRODUCTION

In recent years, many investigations have been conducted to develop and refine techniques for quantitatively determining the amino acids in biological materials. These studies have been of intense interest in the fields of biochemistry, nutrition, medical science, bacteriology, and many other areas. The increasingly wide interest in amino acids and proteins has brought with it the need, and indeed, demand, for accurate, sensitive and rapid amino acid analyses.

Investigations by Drs. MOORE, STEIN, HAMILTON, PIEZ, and others have resulted in accurate and precise methods for amino acid analysis by classical ion-exchange chromatographic techniques. However, over the past eight years, gas–liquid chromatographic (GLC) methods have also reached great refinement, with GLC techniques being widely used for the analysis of lipids, carbohydrates, steroids, various metabolites, pesticides, and many drugs. Similar methods for the routine analysis of amino acids have only recently been reported, since the period 1966 through 1970.

For satisfactory analysis of amino acids by GLC, a complete derivatization of these molecules is essential. Due to the variations in chemical structure and reactivity of the twenty amino acids commonly found in proteins, and other biologically important non-protein amino acids, the quantitative derivatization of all the functional groups under one set of reaction conditions has posed many problems.

Earlier reviews of this area by BLAU[1] and WEINSTEIN[2] discussed in detail various derivatization and chromatographic techniques for the GLC analysis of amino acids. However, prior to 1968, a complete general GLC procedure for the quantitative analysis of the twenty protein amino acids had not been reported.

Extensive research investigations, led by Professor CHARLES W. GEHRKE, resulted in the development of a GLC technique for quantitatively analyzing the twenty protein amino acids as their N-trifluoroacetyl (N-TFA) *n*-butyl ester derivatives. The reaction conditions for quantitatively preparing the amino acid N-TFA *n*-butyl esters of the twenty protein amino acids have been determined[3–7].

Further, complete GLC resolution on a single column of the protein amino acid derivatives has also been extremely difficult to achieve. GEHRKE AND SHAHROKHI[7] reported in 1966 a mixed polyester liquid phase for the separation of the N-TFA *n*-butyl esters of the twenty amino acids, but reproducible elution of arginine, histidine, and cystine was not achieved using this column. STEFANOVIC AND WALKER[8] investigated the use of ethylene glycol adipate (EGA) as a stationary phase for separation of the twenty amino acid N-TFA *n*-butyl ester derivatives, but these workers also did not achieve quantitative elution of arginine, histidine, and cystine.

Investigations were also conducted by MCBRIDE AND KLINGMAN[9] to find a single column which would separate the amino acid N-TFA *n*-butyl esters, and data were reported for all the protein amino acids with the exception of arginine, histidine, and cystine. Studies on the derivatization and chromatography of the amino acid

N-TFA methyl esters were made by DARBRE, BLAU AND ISLAM[10,11]. In their investigations using different mixed siloxane liquid phases, quantitative elution of histidine was not obtained.

In 1968, GEHRKE *et al.*[12] reported on a dual-column chromatographic system, using stabilized ethylene glycol adipate and OV-17 as the liquid phases, from which all twenty of the protein amino acids were quantitatively eluted and separated as their N-TFA *n*-butyl esters. Further, a recent monograph by GEHRKE *et al.*[13] presents in considerable detail macro-semimicro, and micro methods, reagents, sample preparation, instrumental and chromatographic requirements, and sample ion-exchange cleanup for the quantitative GLC analysis of the protein amino acids as their N-TFA *n*-butyl esters.

Refinements of the GLC method have been reported by ROACH *et al.*[14] with regard to the quantitative analysis of histidine, and by ROACH AND GEHRKE[15] on improved performance and reliability of the EGA column of the dual-column system. Conversion of monoacyl histidine to the diacyl derivative on the chromatographic column by injection of trifluoroacetic anhydride has obviated the need for the previously reported *n*-butanol injection. Present studies have shown that histidine (diacyl) can be completely separated from Asp and Phe on the siloxane column of the dual-column system. Further, these investigators have reported that columns containing 0.65 w/w% of stabilized EGA coated on 80/100 mesh AW Chromosorb W, dried at 140° for 12 h, is generally superior to 80/100 mesh AW heat-treated Chromosorb G in terms of resolution, reliability, and ease of preparation[15].

WATERFIELD AND DEL FAVERO[16] reported on the use of silica gel column chromatography for purification of the amino acid N-TFA *n*-butyl esters. After derivatization of the amino acids to the N-TFA *n*-butyl esters, the samples were applied to a silicic acid column, then the amino acids were eluted with diethyl ether. However, for quantitative analyses, difficulties might be expected to be encountered with regard to hydrolysis of the amino acid derivatives during the clean-up procedure.

Recently, the N-trimethylsilyl (TMS) esters of the protein amino acids were extensively investigated by GEHRKE, NAKAMOTO *et al.*[17–19]. This technique offers certain advantages in that trimethylsilylation of the twenty protein amino acids is completed in a single reaction medium, and can be separated on a single chromatographic column. Although this latest method has not yet reached the level of sophistication that has been attained by the N-TFA *n*-butyl ester technique, these researchers have shown that the TMS amino acid derivatives hold great promise as a general and complementary method for routine GLC analysis.

Investigations have also been carried out by GEHRKE and co-workers on the experimental conditions for silylation and GLC analysis of some biologically important groups of molecules: nucleic acid components[20,21], iodo-containing amino acids[22], sulfur-containing amino acids[23], and N-acetylneuraminic acid[24]. An extensive series of studies was made on the exact reaction conditions required for quantitative silylation of each organic class. Detailed methods are presented and data reported on the precision, accuracy, recovery, and application of the methods.

It was the purpose of this investigation to establish the applicability of the developed GLC technique to the quantitative analysis of amino acids as their N-TFA *n*-butyl esters in complex physiological materials, specifically blood plasma and urine. Successful extension of the GLC method into these areas would greatly enhance the

utility of the technique. To this end, ion-exchange methods for cleanup of these complex materials prior to derivatization were studied.

Further, experiments were made to determine the quantitation of the GLC procedure over a wide range of amino acid concentrations, with emphasis on the development of techniques for accurately analyzing microgram and submicrogram amounts of amino acids in physiological substances.

Also, further refinement of the general procedure was studied with regard to the evaluation of various molar excesses of trifluoroacetic anhydride as the acylating reagent. Studies on evaporative losses due to concentration of the N-TFA methyl esters and N-TFA *n*-butyl ester derivatives were also carried out.

These reported GLC methods, developed in the Missouri laboratories were used by the present authors for the analysis of amino acids in the Apollo 11 and 12 returned lunar samples. The authors served as co-investigator and scientists of the NASA-Ames Consortium of principal and co-investigators under the direction of Dr. CYRIL PONNAMPERUMA, senior scientist, of the Ames Research Center, National Aeronautics and Space Administration, Moffett Field, Calif.

EXPERIMENTAL

I. Apparatus

(a) General

A Micro-Tek Model 220 dual hydrogen flame detector gas chromatograph equipped with a Varian Model 30 recorder, and a Varian Aerograph Model 2100 gas chromatograph equipped with a Varian Model 20 recorder were used. A Packard Instrument Co. 7300 Series dual-column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder was also used. A digital readout integrator, Infotronics Model CRS 104 was used for determining the peak areas.

Filters for the hydrogen, air, and nitrogen carrier gas to the chromatographs were obtained from the Packard Instrument Co. and contained Linde 5-A molecular sieve and $CaSO_4$.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump, or the samples were taken just to dryness with a stream of dry, purified nitrogen gas in a 100° constant-temperature sand bath.

Filters containing activated charcoal and $CaSO_4$ were used for purification of the nitrogen gas.

Pyrex 16 × 75 mm glass screw-cap culture tubes with Teflon lined caps (Corning No. 9826) were used as the reaction vessel for the acylation reaction in the macro method and throughout the entire semimicro method.

(b) Macro method

The esterification and interesterification of the amino acids were done in 125-ml $ flat-bottom flasks. For the interesterification, the samples were heated in a 100° constant-temperature oil bath supported by a magnetic hot plate stirrer.

The oil bath used for closed-tube acylation of the amino acid *n*-butyl esters consisted of a 3½ in. × 4 in. × 6 in. aluminum pan supported on a magnetic stirrer to

maintain uniform temperature of the oil. Two 100-W heaters controlled by a Variac were used to maintain the temperature at 150°.

The acylation bath was placed in a hood behind a safety shield (Instruments for Research and Industry-IR²) to provide protection for accidental breakage of the acylation tube.

Automatic repipets (Scientific Products Co.) were attached to 950-ml amber bottles for dispensing $CH_3OH \cdot HCl$, $BuOH \cdot HCl$, and CH_2Cl_2. The top portion of the metal plunger was coated with a silicone to prevent metal contamination.

(c) Micro method

The complete chemical derivatization by the micro technique was carried out in the special micro reaction vials with teflon-lined screw caps (Fig. 1) (Analyctical Biochemistry Laboratories, Columbia, Mo.).

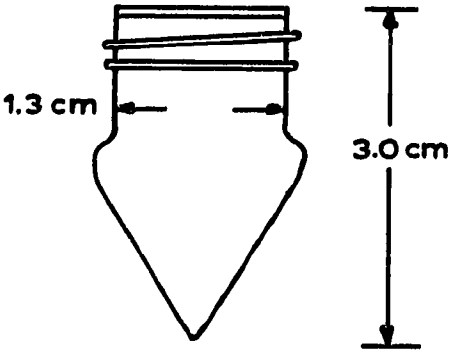

Fig. 1. Reaction vial for the micro method.

The micro samples were heated in a 100° constant temperature sand bath during interesterification.

The solvents were evaporated from the micro samples by placing the sample vials in the sand bath at 100° and directing a stream of dry, purified nitrogen gas into the mouth of the vial, and taking just to dryness.

A Varian Aerograph ultrasonic cleaner was used for mixing of the micro samples.

(d) Ion-exchange cleanup

Amberlite IRA-410 or Dowex 2 (50/100 mesh) resin in the chloride form was used for the removal of picrate from the deproteinized blood plasma.

Amberlite CG-120-H or Dowex 50 (80/100 and 100/200 mesh) resin in the hydrogen form were used to exchange the amino acids present in the physiological samples. These resins can also be used to exchange the amino acids in the presence of picrate from deproteinized samples and to allow the picrate to pass through the column.

Amberlite IRA-410 or Dowex 1 X2 (80/100 and 100/200 mesh)anion-exchange resins in the acetate form were also used for the separation of amino acids from interfering substances present in physiological fluids.

Fischer-Porter pyrex glass columns (Fischer-Porter Co., Warminster, Pa.), 0.9 × 15 cm and 1.5 × 15 cm with sintered glass filters, were packed with the various exchange resins for isolation of the amino acids from interfering materials and picrate.

II. Reagents and materials

(a) General

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y., or Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure. Ribonuclease (Lot No. 26B-1370) was obtained from Sigma Chemical Company, St. Louis, Mo.

Methanol and *n*-butanol were "Baker Analyzed" reagents. The trifluoroacetic anhydride was obtained from Distillation Products Industries, and was an "Eastman Grade" chemical. Acetonitrile, a "Baker Analyzed" reagent of "Nanograde" purity, was stored over drierite in a bottle with a ground glass stopper. Anhydrous HCl, 99.0% minimum purity, was obtained from the Matheson Company, Joliet, Ill.

The methanol, *n*-butanol, and methylene chloride were redistilled from an all-glass system and stored in all-glass inverted top bottles to protect from atmospheric moisture. The methanol was first refluxed over magnesium turnings, and the methylene chloride and *n*-butanol over calcium chloride before distillation. The anhydrous HCl gas was passed through a H_2SO_4 drying tower before bubbling into the *n*-butanol or methanol.

Silanized spun glass, obtained from Analabs, North Haven, Conn., was stored in a desiccator over P_2O_5.

For the ion-exchange cleanup procedures, the following reagents were prepared: 0.02 *N* HCl, 0.01 *N* HCl, 3 *N* HCl, 3 *N* CH_3COOH, 1% picric acid, 3 *N* NH_4OH, 0.1 *N* KOH, and 3 *N* KOH.

(b) Standard stock solutions

Standard amino acid solution. This solution consisted of an aqueous solution (0.1 *N* HCl) containing the twenty protein amino acids at individual concentrations of 2.5 mmoles/l.

n-Butyl stearate internal standard solution (I.S.). This solution contained 2.5 mmoles/l.

(c) GLC columns

Stabilized grade ethylene glycol adipate (EGA) was obtained from Analabs, Inc., North Haven, Conn., and coated on 80/100 mesh Chromosorb G which had been heat-treated as described by GEHRKE *et al.*[12,13]. The EGA column material was packed into 1.5 m × 4 mm I.D. U-shaped glass columns. The recommended packing is described in ref. 15.

The OV-22 siloxane substrate was purchased from Supelco, Inc., Bellefonte, Pa. The support material for the OV-22 column was 80/100 mesh high-performance (HP) Chromosorb G, and the column packing was placed in 1.0 m × 4 mm I.D. U-shaped glass columns.

The column packing was prepared by first adding a known amount of the support material to a 500-ml ribbed round-bottom flask, then adding the solvent used to dissolve the stationary phase until the liquid level was about $\frac{1}{4}$ in. above the support material. The stationary phase was weighed into a small erlenmeyer flask, dissolved in the appropriate solvent (CH_3CN or CH_2Cl_2 for EGA, and CH_2Cl_2 for OV-17 and OV-22) and then transferred to the flask containing the support. The flask containing the support and stationary phase was placed in a 60° water bath, and the solvent was

removed slowly over a period of 45 min with the rotary evaporator under a partial vacuum. The column material was then packed in clean, dry glass columns with gentle tapping to ensure uniform distribution. A plug of silanized spun glass was then placed in each end of the column to hold the column packing in place. The columns were then conditioned as described by GEHRKE *et al.*[12,13].

III. Sample preparation of proteins and biological fluids

(a) Protein hydrolysate

Fifty milligrams of ribonuclease (dried over P_2O_5 at room temperature to a constant weight) were hydrolyzed with 50 ml of constant boiling HCl (6 *N*) for 20 h at 110° in a closed tube under a nitrogen atmosphere. The sample was then dried at room temperature on a rotary evaporator, and the residue transferred to a 50-ml volumetric flask and brought to volume with 0.1 *N* HCl.

(b) Removal of proteins from blood plasma with picrate

Five milliliters of the blood plasma to be deproteinized were placed in a 125-ml erlenmeyer flask, 25 ml of a 1% aqueous picric acid solution were added, and the solution was swirled or stirred with a magnetic stirrer for 5 min. The protein suspension was then centrifuged for 10 min at 3500 r.p.m. and the clear supernatant liquid containing the free amino acids and excess picrate removed by decantation.

(c) Method I—Removal of picrate by anion-exchange chromatography

To remove the picrate, a 25 ml aliquot of the protein-free supernatant from IIIb was passed through a 1.5 × 5 cm column of Amberlite IRA-410 or Dowex 2 (50/100 mesh) resin in the chloride form at the rate of 1 to 2 ml/min. The resin was then washed with two 5-ml portions of 0.02 *N* HCl at a similar rate. Then, the resin was washed with three additional 10-ml portions of 0.02 *N* HCl with the column stopcock completely open. The effluent and washings were collected in a 125-ml ⅋ flat-bottom flask and evaporated to dryness on a rotary evaporator with the flask immersed in a 60° constant-temperature water bath. The residue was then redissolved in 5 ml of 0.1 *N* HCl and placed on a cation-exchange column for exchange separation of the amino acids from interfering materials in the sample.

(d) Preparation of the cation-exchange column

In the following ion-exchange procedures, a 60-fold or greater excess of resin capacity to exchangeable ions placed on the column was maintained.

The resin (Amberlite CG-120-H) was placed in a 500-ml beaker. *Ca.* 3 g of dry resin (1.4-mequiv. capacity) were required for each 0.9 × 15 cm column. The resin was then covered with 3 *N* NH_4OH, swirled for 30 to 60 min, and allowed to settle. The NH_4OH was then removed from the resin by decantation. The process was repeated twice, then washed with d.d. H_2O until approximately neutral.

The resin was regenerated with 3 *N* HCl (three times) as described above, then washed with d.d. H_2O until neutral.

The Fischer and Porter columns were then filled *ca.* half full with the wet resin (*ca.* 3 g dry weight of resin), avoiding any channeling in the column. The column was partially filled with d.d. H_2O and the wet resin was added. The column was washed and drained so that the liquid level was at the resin surface. The sample was then added as follows.

(e) Method II—Cation-exchange cleanup of protein-free blood plasma and urine

The protein- and picrate-free blood plasma which had been dissolved in 5 ml of 0.1 *N* HCl, as described earlier, was carefully placed on the resin and allowed to pass slowly through the column (*ca.* 20 drops/min). The effluent containing the interfering substances was discarded. For cation-exchange cleanup of urine, a 5-ml aliquot of urine was taken to near dryness on a rotary evaporator at 60° with a constant-temperature water bath. The residue was then dissolved in 5 ml of 0.1 *N* HCl, and carefully placed on the resin. The flask was washed three times with 2-ml portions of 0.1 *N* HCl, and these washings were placed on the resin. The solution was then allowed to pass through the column at *ca.* 20 drops/min, and the effluent was discarded. *Caution:* The level of the liquid in the column must not be allowed to fall below the surface of the resin.

Five 5- to 10-ml portions of d.d. H_2O were passed through the column (3 to 5 ml/min) and the washings were discarded.

Five separate 2-ml portions of 3 *N* NH_4OH at *ca.* 1 ml/min, followed by five 5-ml portions of d.d. H_2O, were passed through the column at *ca.* 3 ml/min. The eluate containing the amino acids was collected in a 125-ml flat-bottom flask with $ 24/40 ground glass top. The sample was then taken to dryness at 60° in a constant-temperature water bath by use of a rotary evaporator and stored under refrigeration until analyzed. (Also, the sample may be shell frozen and lyophilized.) Alternatively, the eluate may be collected in a 16 × 75 mm glass culture tube (8-ml volume) which is placed in a 100° constant-temperature sand bath, while directing a stream of dry, purified nitrogen into the tube during elution to aid evaporation.

(f) Method III—Isolation of amino acids from deproteinized samples containing picrate and other interferences

For biological samples to be analyzed by GLC, the amino acids can be exchanged from both picrate and interfering substances in the following single cation-exchange cleanup procedure:

A 0.9 × 7 cm column of Amberlite CG-120-H or Dowex 50 (80/100 or 100/200 mesh) resin in the hydrogen form was prepared.

A 25-ml aliquot of the protein-free supernatant from IIIb was passed through the column at a rate of 1 to 2 ml/min.

Five 5- to 10-ml portions of distilled water were used to wash the resin, and the effluent and washings containing picrate and interfering materials were discarded.

The amino acids were eluted from the resin with five separate 2-ml portions of 3 *N* NH_4OH at 1 to 2 ml/min, followed by five 5-ml portions of distilled water at 3 ml/min.

The alkaline effluent was collected in a 125-ml $ flat-bottom flask and evaporated to dryness as described in IIIc. The sample was then ready for derivatization and analysis by GLC.

For classical ion-exchange chromatographic analysis of the sample, the residue was redissolved in 5.0 ml of 0.1 *N* HCl, transferred to a screw top culture tube with a teflon-lined cap, tightly sealed, and refrigerated until analysis.

(g) Preparation of the anion-exchange column

The resin (Dowex 1 X2) was placed in a 500-ml beaker. *Ca.* 3 g of dry resin

(10 mequiv. capacity) are required for each 0.9 × 15 cm column. The resin was covered with 3 *N* KOH, swirled for 30 to 60 min, and allowed to settle. The KOH was removed from the resin by decantation, and the process was repeated two times. The resin was then washed with d.d. H_2O until approximately neutral.

The resin was regenerated to the acetate form with 3 *N* CH_3COOH as described above, then washed with d.d. H_2O until neutral.

The Fischer and Porter columns were filled *ca.* half full with the wet resin (*ca.* 3 g dry weight of resin), avoiding any channeling in the column. The column was first partially filled with d.d. H_2O, then the wet resin was added. The column was then washed and drained so that the liquid level was at the resin surface. The cation-exchange cleaned urine sample was added to the resin column as follows.

(h) Anion-exchange cleanup of urine

The urine sample cleaned by cation-exchange (procedure IIIe) which had been taken to dryness and redissolved in 5 ml of 0.1 *N* KOH was carefully placed on the resin and allowed to pass slowly through the column at *ca.* 20 drops/min. The effluent was discarded. *Caution:* The level of the liquid in the column must not be allowed to fall below the surface of the resin.

Fifteen 10-ml portions of d.d. H_2O were then passed through the column (3 to 5 ml/min) and the effluent was discarded. (150–250 ml of d.d. H_2O may be required to clean the urine samples, *i.e.* removal of all urea.)

Five separate 3-ml portions of 3 *N* CH_3COOH at *ca.* 20 drops/min, followed by five 5-ml portions of d.d. H_2O, were passed through the column at 3 ml/min. The effluent containing the amino acids was collected in a 125-ml ₹ flat-bottom flask and taken to dryness with a rotary evaporator at 60° in a water bath. The sample was then ready for derivatization and subsequent analysis by GLC.

(i) Recovery studies of the ion-exchange cleanup procedures

To establish the quantitation of the cation- and anion-exchange cleanup procedures for separation of amino acids from interfering materials, known amounts of the protein amino acids were placed on the columns and taken through the described methods. After elution of the amino acids from the cation or anion resin column, an exact amount of ornithine was added as an internal standard, and the amino acids were derivatized to their N-TFA *n*-butyl esters.

Standard samples of amino acids which had not passed through the ion-exchange columns but which contained identical amounts of amino acids and internal standard were also converted to the N-TFA *n*-butyl esters. The percent recovery of each of the protein amino acids taken through exchange columns was then determined by comparison of the GLC analyses of the ion-exchange cleaned samples with the standard samples not passed through the ion-exchange cleanup methods.

To confirm the recovery of the amino acids from physiological materials, known amounts of each of the protein amino acids were added to a stock solution of human urine. The urine solution had previously been analyzed both by GLC and the classical ion-exchange methods. Aliquots of the "spiked" urine solution were then taken through both cation- and anion-exchange cleanup and the amino acids were analyzed by GLC as their N-TFA *n*-butyl esters. *n*-Butyl stearate was used as the internal

standard. Since the amino acid composition of the stock urine solution was known, it was possible to determine the recovery of the added amino acids.

IV. Derivatization of the ion-exchange cleaned physiological fluids

(a) Macro method (5 to 50 mg total)

The effluent from the ion-exchange cleanup procedures containing the amino acids was taken to dryness with the aid of a rotary evaporator and 60° water bath.

To form the methyl esters of the amino acids, 10 ml of methanol·HCl were added to the flasks, stoppered, and stirred at room temperature for 30 min with a magnetic stirrer. The samples were again evaporated to dryness via the rotary evaporator and 60° water bath.

Then, 10 ml of *n*-butanol·HCl were added, along with an appropriate exact aliquot of the *n*-butyl stearate internal standard solution, a drying tube containing $CaSO_4$ was attached to the mouth of the flask, and the flasks were placed in a 100° constant-temperature oil bath and stirred magnetically for 2½ h. The samples were then evaporated to dryness on the rotary evaporator with the aid of a 60° water bath.

The amino acid *n*-butyl esters were then acylated by adding 7 ml of methylene chloride and 1 ml of trifluoroacetic anhydride for each *ca.* 10 mg of amino acids in the sample. The samples were then stirred at room temperature for 15 min.

To complete the acylation, a *ca.* 4 ml aliquot of the solution from the above step was placed in a Corning No. 9826 culture tube. The tube was closed securely with a teflon-lined screw cap, and heated at 150° for 5 min in a constant-temperature oil bath behind a safety shield.

After allowing to cool to room temperature, the samples were then analyzed on the dual-column EGA–OV 17 chromatographic system as described by GEHRKE *et al.*[12,13]. The recommended chromatographic method now used is described in ref. 15.

(b) Semimicro method (0.1 mg to 10 mg total)

An aqueous aliquot of cation-exchange cleaned blood plasma or amino acid mixture containing 0.1 to 10 mg of total amino acids was placed in a Corning No. 9826 glass culture tube, and the samples were evaporated just to dryness by placing the tube in a 100° sand bath while directing a stream of dry, purified nitrogen gas into the heated tube. To ensure complete removal of water from the sample, 0.5 ml of CH_2Cl_2 was then added and evaporated in the same manner.

To form the amino acid methyl esters, 2.0 ml of methanol·HCl were added, the tubes were capped with a teflon-lined screw cap, and mixed by manual inversion and ultrasonic mixing for 15 to 30 sec, then allowed to stand 30 min at room temperature.

The excess methanol·HCl was then evaporated at 100° with nitrogen gas as described above.

The interesterification to form the amino acid *n*-butyl esters was performed by adding 2.0 ml of *n*-butanol·HCl, mixing as described above and the tightly capped samples were then heated at 100° for 2½ h in the sand bath.

The excess *n*-butanol·HCl was then evaporated from the sample with the 100° sand bath and nitrogen gas as described above.

The amino acid *n*-butyl esters were acylated by adding 0.8 ml of CH_2Cl_2 and 0.2 ml of TFAA, the tube was then securely capped, and the solution was mixed by manual inversion and ultrasonic mixing. To complete the acylation, the tubes were

placed in a 150° constant temperature oil bath for 5 minutes. After allowing to cool, the samples were then analyzed by the dual-column EGA–OV-17 chromatographic system.

(c) Micro method (2 μg to 200 μg total)

An aqueous aliquot of an amino acid mixture containing 2 μg of total amino acids was placed in the special screw cap reaction vial (Fig. 1), and evaporated just to dryness using the 100° sand bath and a stream of dry, purified N_2 gas as described in the previous section. Azeotropic removal of water was completed by adding 100 μl of CH_2Cl_2 and evaporating just to dryness in the above manner. To ensure complete removal of water, this step was repeated.

The amino acid methyl esters were formed by addition of 100 μl of methanol · HCl to the reaction vial. The vial was capped with a teflon-lined screw cap, then the sample was mixed ultrasonically for 15 to 30 sec and allowed to esterify at room temperature for 30 min with mixing at 10-min intervals.

The methanol · HCl was evaporated at 100° with nitrogen gas as described above.

For interesterification to the *n*-butyl esters, 100 μl of *n*-butanol · HCl were added to the reaction vial, and mixed ultrasonically. The solution was then heated at 100° for 2½ h in the sand bath, with ultrasonic mixing at 30-min intervals.

The *n*-butanol · HCl was then evaporated at 100° with a sand bath and nitrogen gas as described above.

The amino acid *n*-butyl esters were acylated by addition of 60 μl of CH_2Cl_2 and 20 μl of TFAA, ultrasonic mixing, and acylation at 150° for 5 min in an oil bath. The samples were then ready for GLC analysis.

V. TFAA molar excess

During chromatography of the amino acid N-TFA *n*-butyl esters on a polyester liquid phase (EGA), it had previously been noted that the chromatographic peak due to trifluoroacetic acid interfered with the quantitation of the most volatile amino acid derivatives (alanine, glycine). Therefore, experiments were conducted to determine the optimum molar ratio of TFAA/amino acids that would result in the most accurate and precise analysis of the twenty protein amino acids.

Equal aliquots of the standard amino acid solution were derivatized to their N-TFA *n*-butyl esters as described in section IVc and acylated at 150° for 5 min using 4, 10, 20, 50, and 100 molar excess of TFAA/amino acids. This experiment was repeated twice independently, with duplicate GLC analyses being made on each sample.

VI. Loss of derivatives on evaporation

To study the comparative losses during evaporation of the N-TFA methyl esters and N-TFA *n*-butyl esters of the protein amino acids, the following procedures were used.

(a) N-TFA methyl esters

A 1-ml aliquot of the 2.5 m*M* standard amino acid solution was placed in a 16 × 75 mm glass culture tube and dried at 100° in a sand bath with a stream of dry, purified nitrogen gas directed into the mouth of the tube.

To form the amino acid methyl esters, 1 ml of methanol · HCl was added to the

culture tube, closed, and heated at 100° in the sand bath for 1 h. The sample was then dried with nitrogen gas and the 100° sand bath as described above.

The N-trifluoroacetyl methyl esters were then formed by adding 1.8 ml of CH_2Cl_2 and 0.2 ml of TFAA (50 molar excess) and heating the tightly capped tubes for 5 min at 150° in an oil bath.

The derivatized sample was then analyzed by GLC in triplicate.

The remaining derivatized sample was then divided into two equal parts and taken to dryness with nitrogen gas, one at 100° and the other at 25° to observe the effect of evaporation temperature on loss of the amino acid N-TFA methyl esters.

Both samples were again reacylated as in VIa, and duplicate chromatographic analyses were made on each sample.

(b) N-TFA n-butyl esters

A 1-ml aliquot of the 2.5 m*M* standard amino acid solution was placed in a 16 × 75 mm glass culture tube and dried at 100° in the sand bath under a stream of nitrogen gas.

The amino acid methyl esters were formed as in VIa of the preceding section and again dried with the aid of nitrogen gas and the 100° sand bath.

One milliliter of *n*-butanol · HCl was then added to the tube and the tightly capped tube was heated at 100° for 2½ h in the sand bath. The sample was then taken to dryness on the 100° sand bath with nitrogen gas as previously described.

To acylate the amino acid *n*-butyl esters, 1.8 ml of CH_2Cl_2 and 0.2 ml of TFAA (50 molar excess) were added, and the tube was heated at 150° for 5 min in a constant-temperature oil bath.

Duplicate chromatographic analyses were then made on the derivatized sample.

The remaining derivatized sample was then taken to dryness at 100° on the sand bath with nitrogen gas, reacylated as above, and again duplicate chromatographic analyses were performed.

The above sample was then dried under nitrogen gas at 25°, reacylated again as above, and again two chromatographic analyses were made.

RESULTS AND DISCUSSION

Concentration of N-TFA amino acid esters

The effect of concentrating the amino acid N-TFA *n*-butyl esters at various temperatures with regard to loss of the amino acid derivatives was studied. Also, a similar study was carried out with the N-TFA methyl esters. The acylated samples were concentrated under dry, purified nitrogen gas at temperatures of 25° and 100°. Table I presents the relative response factor data obtained from the GLC analysis of the N-TFA methyl ester derivatives. No attempt was made to identify the chromatographic peaks in this case, and a relative response factor for each peak was calculated relative to the thirteenth peak, which was arbitrarily assigned a value of unity. It is seen from these data that losses of up to 100% of the N-TFA methyl ester derivatives occurred during evaporation at 25°. Table II presents the corresponding data for the N-TFA *n*-butyl derivatives. It is apparent from these data that the N-TFA *n*-butyl esters sustain only minor losses when evaporated at 100° and even lower relative losses when dried at 25°.

TABLE I

EFFECT OF EVAPORATION ON THE N-TFA METHYL ESTER DERIVATIVES[a]

Peak No.	*Relative response factors*[b]							
	Original[c]			*Av.*	*25° evaporation*		*Av.*	*Relative loss (%)*
1	—[d]	—[d]	—[d]	—[d]	—	—	—	—
2	—[d]	—[d]	—[d]	—[d]	—	—	—	—
3	0.15	0.16	0.16	0.16	—	—	—	100
4	0.45	0.45	0.43	0.44	0.15	0.15	0.15	66
5	0.64	0.65	0.63	0.64	0.32	0.32	0.32	50
6	0.55	0.53	0.53	0.54	0.31	0.31	0.31	43
7	0.47	0.47	0.49	0.48	0.25	0.25	0.25	48
8	0.35	0.35	0.37	0.36	0.24	0.24	0.24	33
9	0.40	0.41	0.40	0.40	0.33	0.33	0.33	17
10	0.64	0.64	0.68	0.65	0.54	0.53	0.54	17
11	0.42	0.43	0.43	0.43	0.44	0.44	0.44	0
12	1.18	1.18	1.13	1.16	1.13	1.13	1.13	0
13	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
14	0.63	0.63	0.63	0.63	0.58	0.58	0.58	9
15	0.50	0.51	0.52	0.51	0.40	0.39	0.40	—

[a] Evaporated just to dryness under nitrogen gas.
[b] Calculated relative to peak No. 13, which was assigned the value of unity.
[c] Analyzed before evaporation.
[d] Not resolved.

LE II

CT OF EVAPORATION ON THE N-TFA *n*-BUTYL ESTER DERIVATIVES[a]

no acid	*Relative response factors*[b]										
	Original[c]		*Av.*	*100° evaporation*		*Av.*	*Relative loss (%)*	*25° evaporation*		*Av.*	*Relative loss (%)*
ine	0.45	0.44	0.45	0.41	0.40	0.41	9	0.37	0.37	0.37	10
ne	0.51	0.51	0.51	0.46	0.46	0.46	10	0.42	0.42	0.42	9
ine	0.41	0.40	0.41	0.37	0.37	0.37	10	0.34	0.35	0.35	5
ucine	0.62	0.61	0.62	0.58	0.57	0.58	6	0.53	0.53	0.53	9
ine	0.64	0.63	0.64	0.59	0.59	0.59	8	0.59	0.58	0.59	0
ine	0.70	0.70	0.70	0.67	0.68	0.68	3	0.67	0.66	0.67	1
onine	0.58	0.58	0.58	0.54	0.54	0.54	7	0.54	0.52	0.53	2
ne	0.58	0.57	0.58	0.54	0.53	0.54	7	0.56	0.53	0.55	0
ionine	0.12	0.11	0.12	0.09	0.07	0.08	34	0.12	0.08	0.10	0
roxyproline	0.76	0.75	0.76	0.75	0.75	0.75	1	0.75	0.73	0.74	1
ylalanine	1.11	1.11	1.11	1.10	1.10	1.10	1	1.10	1.11	1.11	0
rtic acid	0.83	0.83	0.83	0.83	0.82	0.83	0	0.85	0.83	0.84	0
amic acid	1.00	1.00	1.00	1.00	1.00	1.00	0	1.00	1.00	1.00	0
sine	0.94	0.95	0.95	0.94	0.94	0.94	1	0.94	0.93	0.94	0
thine	0.78	0.76	0.77	0.76	0.76	0.76	1	0.74	0.75	0.75	1
ne	0.88	0.87	0.88	0.84	0.84	0.84	5	0.83	0.83	0.83	1

[a] Evaporated just to dryness under nitrogen gas.
[b] Calculated relative to glutamic acid, which was assigned the value of unity.
[c] Analyzed before evaporation.

TFAA molar excess

To refine further the developed GLC technique for the quantitative analysis of amino acids, various molar ratios of TFAA/total amino acids were evaluated. Molar ratios of 4, 10, 20, 50, and 100 of TFAA/total amino acids during the acylation step were used.

Two independent samples were prepared at each of the TFAA concentrations, and duplicate GLC analyses were made on each sample. Table III presents the data

TABLE III

THE INFLUENCE OF EXCESS TFAA ON THE RMR VALUES FOR THE TAB DERIVATIVES OF THE AMINO ACIDS[a]

Amino acid	*4-molar*[b] *excess*	*10-molar*[b] *excess*	*20-molar*[b] *excess*	*50-molar*[b] *excess*	*100-molar*[b] *excess*
Alanine	0.44	0.45	0.49	0.48	0.49
Valine	0.52	0.54	0.61	0.63	0.62
Glycine	0.39	0.40	0.39	0.41	0.40
Isoleucine	0.63	0.68	0.74	0.73	0.74
Leucine	0.78	0.77	0.78	0.74	0.75
Proline	0.70	0.72	0.68	0.69	0.70
Threonine	0.54	0.59	0.61	0.63	0.65
Serine	0.56	0.55	0.54	0.56	0.55
Methionine	0.64	0.59	0.63	0.63	0.62
Hydroxyproline	0.64	0.75	0.76	0.73	0.75
Phenylalanine	1.08	1.12	1.14	1.11	1.12
Aspartic acid	0.85	0.84	0.86	0.83	0.84
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.35	0.97	0.94	0.96	0.99
Ornithine	0.73	0.76	0.74	0.73	0.72
Lysine	0.78	0.85	0.84	0.86	0.87

[a] Absolute molar excess in each case.

[b] Each of these values represent the average of duplicate analyses of two independent samples.

obtained from these experiments. A 50-molar or greater excess of TFAA/total amino acids was found to be best with regard to accuracy, precision, and stability of the acylated samples. A larger excess of TFAA created problems with regard to sample volume especially in samples analyzed at the micro method level; thus molar ratios larger than 50:1 of TFAA/total amino acids were not used.

GLC analysis of microgram and submicrogram amounts of amino acids

To determine the applicability of the developed GLC technique for the analysis of microgram and submicrogram amounts of amino acids in physiological fluids or proteins, aliquots of the standard amino acid solution containing microgram quantities of each amino acid were derivatized according to the micro method and analyzed. In these experiments, the micro reaction vials were used, with reaction volumes of *ca.* 80 μl. In all cases it was necessary to maintain solubility of the sample by retaining a liquid phase during acylation.

A typical chromatogram obtained from the derivatization and GLC analysis of 2.5 μg of each amino acid is presented in Fig. 2, with each peak representing *ca.* 150 ng of amino acid injected. Fig. 3 presents the chromatogram obtained from the analysis

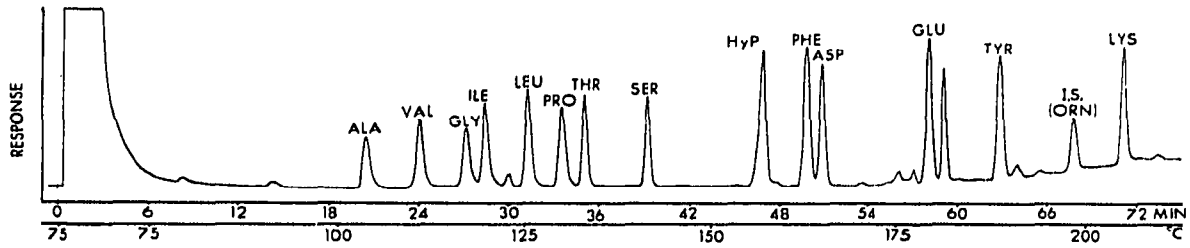

Fig. 2. Chromatogram obtained on GLC of 2.5 μg of each of the amino acid N-TFA *n*-butyl esters. Conditions: Varian Aerograph 2100; column, 0.325 w/w% EGA on 80/100 mesh AW heat-treated Chromosorb G, 1.5 m × 4 mm I.D., glass; nitrogen flow rate, 70 ml/min; temperature, programmed at a rate of 2°/min, initial temperature 75°. Sample, 80 μl. Each peak represents *ca.* 150 ng. Internal standard, ornithine.

of a sample containing only 1 μg of each amino acid, or 20 μg of total amino acids, with each chromatographic peak corresponding to *ca.* 60 ng. The relative standard deviation was found to be in the range of 2 to 10%. Fig. 4 presents data on a submicrogram analysis of amino acids. In this experiment only 0.1 μg of each amino acid was derivatized, with each peak representing *ca.* 6 ng of amino acid. By concentrating the

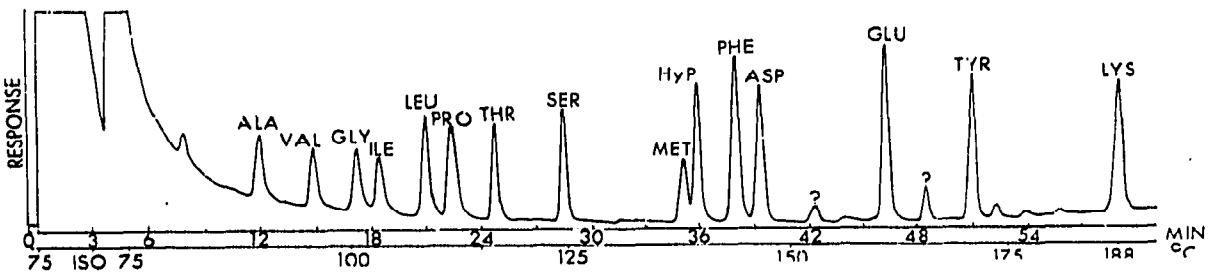

Fig. 3. Chromatogram obtained on GLC of 1 μg of each of the amino acid N-TFA *n*-butyl esters. Temperature, programmed at a rate of 2°/min up to 195°, initial temperature, 75°. For further conditions, see the legend to Fig. 2. Sample, 80 μl. Volume injected, 5 μl. Each peak represents *ca.* 60 ng. Sensitivity, 1 × 2.

final 80 μl of acylation solution to 20 μl, and injecting 8 μl, the amount of starting material could be reduced to 10 ng or less of each amino acid and a semiquantitative analysis could still be achieved.

Analysis of human blood plasma

GLC analyses of deproteinized human blood plasma indicated that further

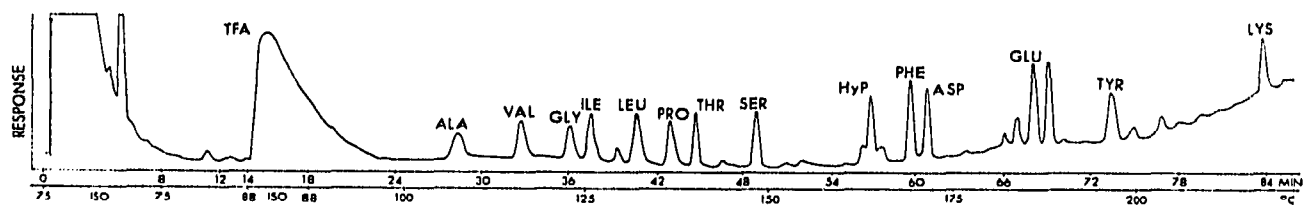

Fig. 4. Chromatogram obtained on GLC of submicrogram amounts of the amino acid N-TFA *n*-butyl esters. Temperature, programmed at a rate of 2°/min, initial temperature 75°. For further conditions, see the legend to Fig. 2. Sample, 0.1 μg of each amino acid in 80 μl. Volume injected, 5 μl. Each peak represents *ca.* 6 ng. Sensitivity, 0.1 × 2.

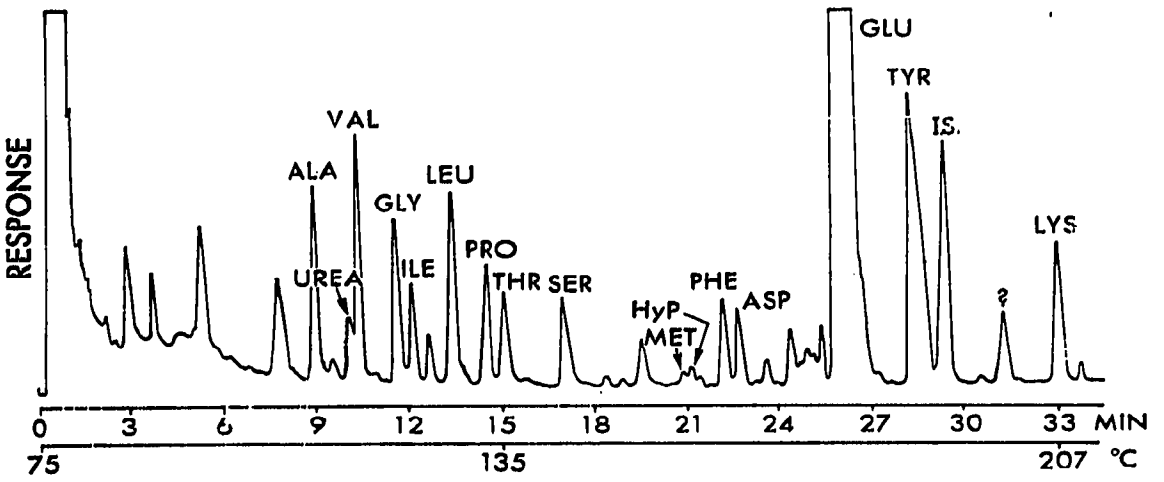

Fig. 5. Chromatogram obtained on GLC of human blood plasma amino acid N-TFA *n*-butyl esters not cleaned by cation exchange. Temperature, programmed at a rate of 4°/min, initial temperature 75°. For further conditions, see the legend to Fig. 2. Sample, *ca.* 6 ml of plasma in a final volume of 2.5 ml. Volume injected, 5 μl (*ca.* 3.5 μg total amino acids). Internal standard, *n*-butyl stearate. Final temperature, 210°.

TABLE IV

AMINO ACID ANALYSIS OF HUMAN BLOOD PLASMA

Amino acid	*Mg per 100 ml of plasma*								
	Gas–liquid chromatography[a,f]				*Ion-exchange chromatography*		*Average*		
	Macro		*Micro*				*Macro*	*Micro*	*IE*
Alanine	3.12	3.12	2.92	2.97	3.45	3.39	3.12	2.95	3.4
Valine	3.24	3.48	3.21	3.51	3.25	3.24	3.36	3.36	3.2
Glycine	2.58	2.72	2.54	2.88	2.68	2.69	2.65	2.71	2.6
Isoleucine[b]	1.41	1.38	1.67	1.55	1.19	1.24	1.40	1.61	1.2
Leucine	2.49	2.68	2.65	2.15	2.73	2.56	2.59	2.40	2.6
Proline[c]	2.69	2.63	2.99	2.84	2.92	2.10	2.66	2.92	2.5
Threonine	2.07	2.02	2.20	2.22	1.50	1.60	2.05	2.21	1.5
Serine	1.80	1.76	1.74	1.81	1.42	1.46	1.78	1.78	1.4
Methionine	0.61	0.62	0.75	0.71	0.37	0.32	0.62	0.73	0.3
Phenylalanine	0.92	0.92	0.97	0.86	0.98	0.95	0.92	0.92	0.9
Aspartic acid[d]	1.29	1.30	1.29	1.23	0.70	0.66	1.30[d]	1.26	0.6
Glutamic acid[d]	3.35	3.34	3.22	3.30	2.12	2.08	3.35[d]	3.26	2.1
Tyrosine	0.98	0.98	0.94	0.99	0.93	0.86	0.98	0.97	0.9
Ornithine	2.01	2.05	2.30	2.19	1.74	1.72	2.03	2.25	1.7
Lysine	3.43	3.60	3.38	3.21	3.62	3.60	3.52	3.30	3.6
Arginine	1.20	1.32	1.26	1.17	1.58	1.61	1.27	1.22	1.6
Histidine[e]	1.01	1.32	1.19	1.09	1.93	1.93	1.17	1.14	1.9
Tryptophan[e]	0.63	0.57	0.66	0.69			0.60	0.68	
Cystine	0.45	0.43	0.49	0.54	T	T	0.44	0.52	T
							35.81	36.19	32.1

[a] Cleaned by cation exchange prior to derivatization.
[b] GLC values include *allo*-isoleucine.
[c] The lack of ion-exchange precision is due to poor separation of Pro and Cit.
[d] GLC values include Asp(NH_2) and Glu(NH_2).
[e] His and Trp not separated by ion-exchange.
[f] N-TFA *n*-butyl esters.

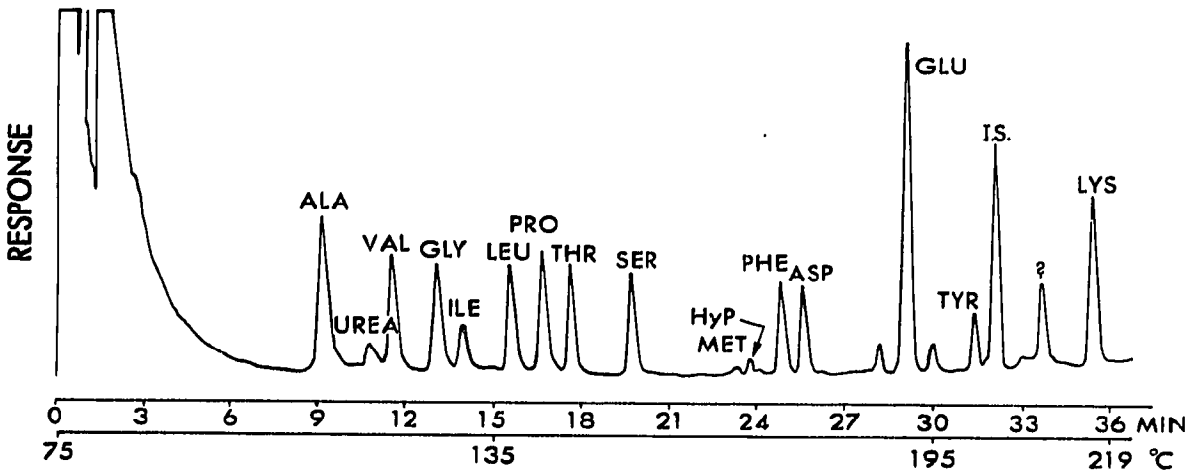

Fig. 6. Chromatogram obtained on GLC of human blood plasma amino acid N-TFA *n*-butyl esters cleaned by cation exchange. For further information, see the legend to Fig. 5. Final temperature, 220°.

sample cleanup was necessary to achieve quantitative amino acid analysis. Fig. 5 shows a typical chromatogram for amino acids in an uncleaned deproteinized blood plasma on an EGA column. In order to remove the extraneous substances in blood plasma which interfere with GLC analysis, the cation-exchange cleanup procedure

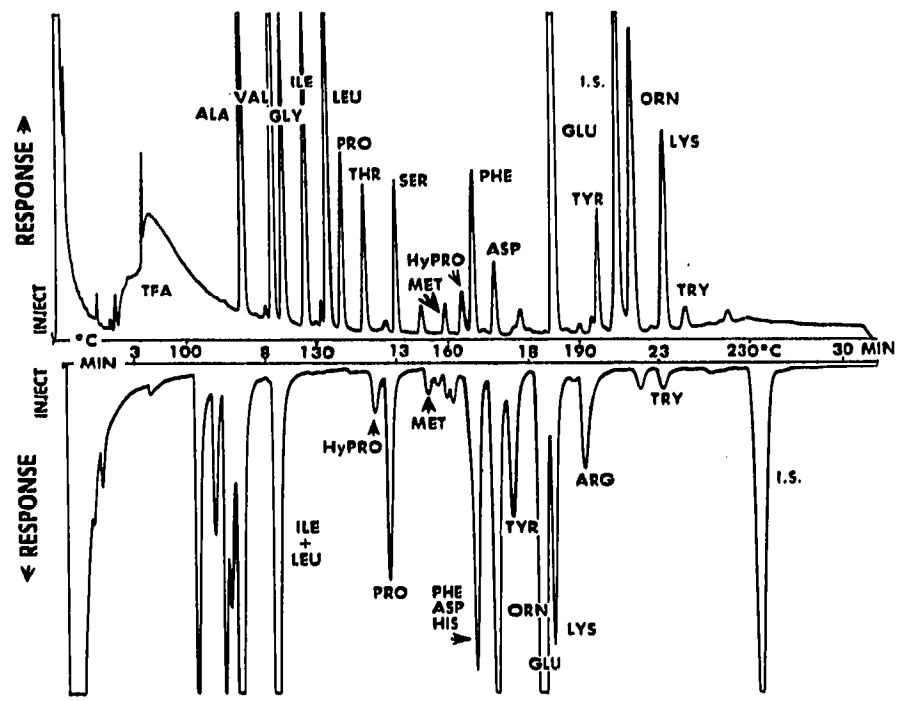

Fig. 7. GLC analysis of bovine blood plasma. 10.0 ml of plasma deproteinized with 40 ml of 1% picric acid, cation-exchange-cleaned. Final acylation volume, 2 ml; injected, 5 μl; *ca.* 5 μg total amino acid injected. Attenuation, 8 × 10^{-10} AFS; initial temperature, 70°; program rate, 6°/min; final temperature, 230°. Upper part: Column, 0.65 w/w% EGA on 80/100 mesh A.W. Chromosorb W, 1.5 m × 4 mm I.D. glass. Lower part: column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate.

was studied. After passing the deproteinized plasma through a small cation-exchange column, the sample was again chromatographed on an EGA column and found to be suitable for quantitative analysis by GLC (Fig. 6). Fig. 7 shows a complete amino acid analysis of cation-exchange cleaned blood plasma on both EGA and OV-17 columns.

To investigate the quantitation of the micro method for the analysis of biological samples, duplicate aliquots of a stock solution of deproteinized human blood plasma were analyzed by GLC at both the macro and micro levels, and also by the classical ion-exchange technique. The samples analyzed by GLC were cleaned by cation exchange prior to derivatization to the N-TFA *n*-butyl esters. The classical ion-exchange analyses were made without prior cleanup. The data obtained from each of these experiments are presented in Table IV and give comparisons for the macro analyses (*ca.* 4 mg of total amino acids), micro analyses (*ca.* 200 μg of total amino acids), and classical ion-exchange analyses. All of the data were in good agreement. Also, it is noted from this table that quantitative recovery of the amino acids from the cation-exchange cleanup column was achieved.

Recovery from the cation-exchange cleanup column

To establish further the quantitation of the cation-exchange cleanup technique, the recovery of each of the protein amino acids from the column was established by taking known amounts of each of the amino acids through the cation cleanup pro-

TABLE V

RECOVERY OF AMINO ACIDS FROM CATION-EXCHANGE CLEANUP

Amino acid	*Milligrams of amino acids*				*Recovery (%)*
	Added	*Found*[a,b,c]		*Av.*	
Alanine	2.23	2.21	2.23	2.22	99.6
Valine	2.93	2.90	2.81	2.85	97.3
Glycine	1.88	1.88	1.95	1.92	102.1
Isoleucine	3.28	3.23	3.11	3.17	96.6
Leucine	3.28	3.22	3.31	3.26	99.4
Proline	2.88	2.83	2.91	2.87	99.7
Threonine	2.98	2.95	2.84	2.89	97.0
Serine	2.63	2.57	2.63	2.60	98.9
Methionine	3.73	3.80	3.87	3.84	102.9
Hydroxyproline	3.27	3.21	3.27	3.24	99.1
Phenylalanine	4.13	4.08	3.89	3.98	96.4
Aspartic acid	3.32	3.27	3.32	3.30	99.4
Glutamic acid	3.68	3.63	3.63	3.63	98.6
Tyrosine	4.53	4.46	4.46	4.46	98.5
Lysine	3.66	3.60	3.66	3.63	98.6
Arginine	4.36	4.10	4.24	4.17	95.6
Tryptophan	5.10	4.86	4.99	4.93	96.7
Histidine[d]	3.88	3.74	3.71	3.73	96.1
Cystine	6.01	5.84	5.93	5.89	98.0
	67.76			66.58	Av. 98.5

[a] Analyses by GLC as the N-TFA *n*-butyl esters.
[b] Ornithine as internal standard.
[c] Each value represents an independent analysis.
[d] Analyzed as the diacyl derivative after injection of 4 μl of TFAA[14].

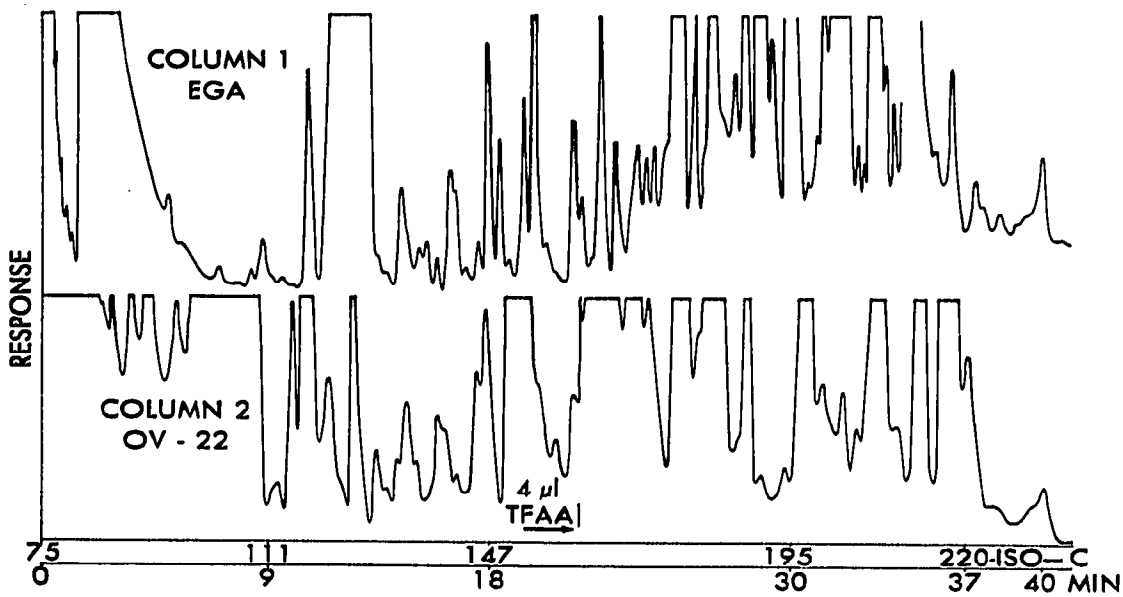

Fig. 8. Chromatogram obtained on GLC of human urine amino acid N-TFA *n*-butyl esters. Not cleaned by cation exchange on two different columns. Conditions: Column 1—0.65 w/w% EGA on 80/100 mesh dried (140°, for 12 h) AW Chromosorb W, 1.5 m × 4 mm I.D., glass. Column 2—1.5 w/w% OV-22 on 80/100 mesh HP Chromosorb G, 1.0 m × 4 mm I.D., glass. Temperature, programmed at a rate of 4°/min up to 220°, initial temperature 75°. Sample, 4 ml (*ca.* 4 mg amino acids in 2.5 ml final acylation volume). Volume injected, 8 µl (*ca.* 13 µg total amino acids).

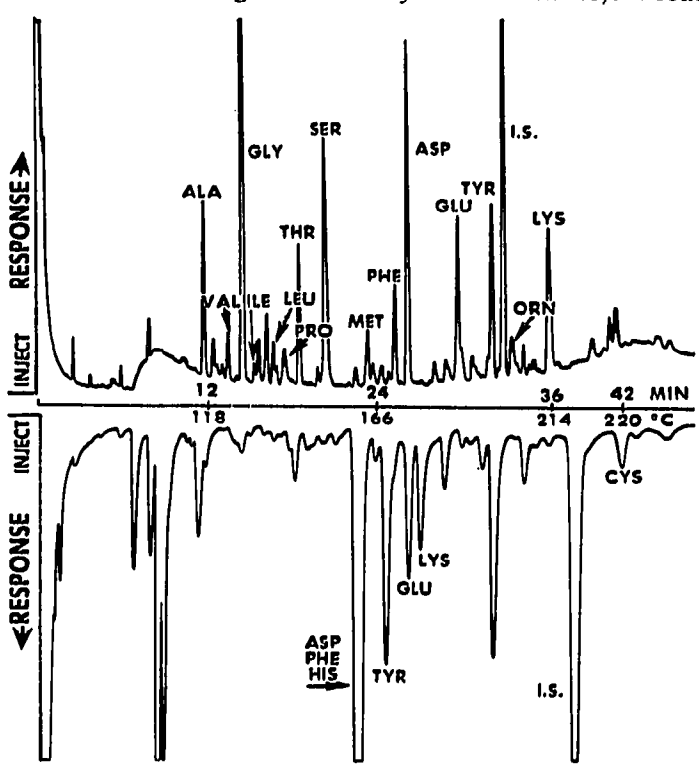

Fig. 9. GLC analysis of human urine. 4.5 ml of human urine hydrolyzed with 1 *N* HCl at 110° for 2 h. Final acylation volume, 2 ml; injected, 5 µl, *ca.* 2 µg of total amino acid injected. Initial temperature, 70°; program rate, 4°/min; final temperature, 220°; attenuation, 8 × 10^{-10} AFS. Upper part: column, 0.65 w/w% EGA on 80/100 mesh A.W. Chromosorb G, 1.5 m × 4 mm I.D. glass. Lower part: column, 1.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 1 m × 4 mm I.D. glass. Internal standard, *n*-butyl stearate.

cedure. The eluates from the columns were derivatized and analyzed by GLC. Also, identical samples which had not been passed through the cleanup columns were analyzed in the same manner, and the per cent recovery of each amino acid through the cation-exchange column was determined by comparison of these analyses. These data are presented in Table V, and indicate that no losses occurred during cation-exchange cleanup of biological samples.

GLC analysis of human urine

Initial GLC analyses of uncleaned human urine revealed that an efficient method of removing extraneous substances was essential. A chromatogram of the N-TFA *n*-butyl derivatives of uncleaned human urine is shown in Fig. 8.

Aliquots of a stock urine solution, cleaned by both the cation- and anion-exchange procedures, were analyzed by GLC and the classical ion-exchange techniques. A GLC chromatogram of the cleaned urine is shown in Fig. 9. Also, classical ion-exchange analyses were made on aliquots of unhydrolyzed stock solution and on

TABLE VI

AMINO ACID ANALYSIS OF HUMAN URINE[a]

Amino acid	*Mg per 100 ml of urine*										
	Gas–liquid chromatography[b]			*Ion-exchange chromatography*[d]				*Average*		*RSD*[c] (%	
				Non-hydrolyzed		*Hydrolyzed*[e]		*GLC*	*IE*	*GLC*	*I.*
Alanine	2.82	2.64	2.78	2.39	2.75	2.52	2.58	2.75	2.56	2.79	
Valine	0.66	0.67	0.59	0.60	0.60	0.78	0.75	0.64	0.68	5.40	1
Glycine	16.52	16.58	16.64	17.18	17.19	17.78	17.53	16.58	17.42	0.03	
Isoleucine	T[f]	0.18	0.24	0.23	0.28	0.24	0.22	0.21	0.24		
Leucine	0.78	0.68	0.82	—	0.70	0.77	0.76	0.76	0.74	7.70	
Proline	T	T	T	T	T	T	T	T	T		
Threonine	3.02	2.98	3.08	2.64	2.88	3.07	2.51	3.03	2.78	1.40	
Serine	5.56	5.62	5.60	5.39	5.28	5.28	5.43	5.59	5.35	0.40	
Methionine	0.20	0.47	0.50	0.41	0.34	0.31	—	0.39	0.35		
Hydroxyproline	0.00	T	T	0.00	0.00	0.00	0.00	T	0.00		
Cysteine	0.00	0.00	T	T	T	T	T	T	T		
Phenylalanine	1.63	1.71	1.66	1.68	1.81	1.92	1.77	1.67	1.80	3.70	
Aspartic acid	5.88	5.74	5.62	0.52	0.71	5.00	5.30	5.74	5.15	1.40	
Glutamic acid	10.31	10.20	10.16	0.68	0.72	10.15	9.69	10.22	9.92	0.60	
Tyrosine	3.68	3.80	3.76	3.41	3.45	3.31	3.44	3.75	3.40	0.13	
Lysine	3.98	4.20	4.12	—	—	3.92	4.50	4.10	4.21	2.20	
Arginine	0.21	0.25	0.16	0.11	0.14	0.20	0.26	0.21	0.18		
Histidine	26.51	25.46	27.30	27.91	26.65	29.11	—	26.42	27.89	0.78	
Tryptophan	5.62	5.49	5.68	g	g	g	g	5.60	g	1.40	
Cystine	0.08	0.13	0.09	0.11	0.14			0.10	0.13		
								87.76	86.91	2.15[h]	

[a] Cleaned by cation and anion exchange prior to analysis.
[b] Each an independent analysis.
[c] Relative standard deviation, %.
[d] Each an independent analysis, norleucine as internal standard.
[e] Hydrolyzed 2 h at 110° under nitrogen gas in 1 *N* HCl.
[f] Trace.
[g] Not resolved from histidine.
[h] Average RSD, %. Note conversion of $Asp(NH_2)$ and $Glu(NH_2)$ to Asp and Glu on hydrolysis.

aliquots subjected to a mild hydrolysis. The data from these analyses are presented in Table VI and the results are seen to be in good agreement. If the concentrations of the amino acids in urine samples are usually divergent then two analyses of a sample should be made using internal standards at high and low concentrations approximating that of the amino acids in the sample. In this way, the precision and accuracy of the analysis will be greatly improved.

To establish the quantitation of the anion-exchange cleanup method, known amounts of each of the protein amino acids were taken through the anion-exchange cleanup procedure as described earlier, and the percent recovery of each determined. These data are presented in Table VII, and the recovery was quantitative. To confirm

TABLE VII

RECOVERY OF AMINO ACIDS FROM ANION-EXCHANGE CLEANUP

Amino acid	*Milligrams of amino acids*					*Recovery (%)*	*RSD (%)*
	Added	*Found*[a,b,c]			*Av.*		
Alanine	0.891	0.917	0.911	0.913	0.914	102.6	1.07
Valine	1.171	1.210	1.203	1.191	1.201	102.6	0.80
Glycine	0.751	0.738	0.746	0.741	0.742	98.9	0.55
Isoleucine	1.312	1.281	1.326	1.423	1.343	102.4	5.40
Leucine	1.312	1.278	1.285	1.293	1.285	97.9	0.58
Proline	1.151	1.126	1.113	1.213	1.151	100.0	4.72
Threonine	1.191	1.190	1.241	1.238	1.223	102.7	2.34
Serine	1.051	1.091	1.073	1.071	1.078	102.6	1.02
Methionine	1.492	1.436	1.451	1.479	1.455	97.5	1.50
Hydroxyproline	1.312	1.343	1.351	1.336	1.343	102.4	0.56
Phenylalanine	1.652	1.692	1.691	1.621	1.668	101.0	2.44
Aspartic acid	1.331	1.374	1.362	1.371	1.369	102.8	0.46
Glutamic acid	1.470	1.491	1.498	1.499	1.496	101.8	0.29
Tyrosine	1.612	1.642	1.651	1.648	1.647	102.2	0.28
Lysine	1.462	1.497	1.492	1.501	1.497	102.4	0.30
Arginine	1.742	1.745	1.773	1.786	1.768	101.5	1.19
Tryptophan	2.042	2.091	2.084	2.080	2.085	102.1	0.27
Histidine	1.552	1.501	1.496	1.510	1.502	96.8	0.47
Cystine	2.403	2.381	2.380	2.369	2.377	98.9	0.28
	26.900				27.140	Av 101.0	Av 1.29

[a] Analyses by GLC as the N-TFA *n*-butyl esters.
[b] Ornithine as internal standard.
[c] Each value represents an independent analysis.

further the quantitation of the entire cleanup procedure through both cation- and anion-exchange columns known amounts of each of the protein amino acids were added to the "stock" urine solution. Then, aliquots of this solution were taken through both the cation- and anion-exchange cleanup, and analyzed by GLC. The recovery of the amino acids from the urine is given in Table VIII, and was quantitative.

COMMENTS ON THE GLC METHOD

The injection port should be maintained at 200°; the detector at 250°.

Moisture must be excluded from both the sample during derivatization and the chromatographic column.

TABLE VIII

RECOVERY OF AMINO ACIDS FROM URINE

Amino acid	*Milligrams of amino acids*					*Recovery (%)*	*RSD (%)*
	Added[a]	*Found*[b,c]			*Av.*		
Alanine	2.23	2.17	2.27	2.30	2.25	100.9	3.03
Valine	2.93	2.99	2.81	2.84	2.88	98.3	3.37
Glycine	1.88	1.79	1.94	1.99	1.91	101.6	5.47
Isoleucine	3.28	3.35	3.39	3.42	3.39	103.4	1.06
Leucine	3.28	3.10	3.19	3.20	3.16	96.3	1.76
Proline	2.88	2.74	2.76	2.80	2.77	96.1	1.11
Threonine	2.98	2.91	2.94	2.95	2.93	98.3	0.72
Serine	2.63	2.65	2.67	2.69	2.67	101.5	0.75
Methionine	3.73	3.90	3.89	3.85	3.88	104.0	0.68
Hydroxyproline	3.28	3.41	3.47	3.48	3.45	105.2	1.10
Phenylalanine	4.13	4.10	4.06	4.04	4.06	98.3	0.78
Aspartic acid	3.33	3.17	3.11	3.19	3.42	94.8	1.22
Glutamic acid	3.68	3.60	3.59	3.62	3.60	97.8	0.44
Tyrosine	4.53	4.37	4.39	4.36	4.37	96.5	0.36
Lysine	3.66	3.69	3.74	3.75	3.73	101.9	0.87
Histidine	3.88	3.78	3.69	3.85	3.77	97.2	2.13
Arginine	4.36	4.30	4.51	4.48	4.43	101.6	2.56
Tryptophan	5.11	4.70	4.78	4.64	4.71	92.2	1.49
Cystine	6.01	6.18	6.20	6.29	6.22	103.5	0.94
	67.79				66.60	Av. 99.44	Av. 1.57

[a] Known amounts were added to a stock sample of human urine, then cleaned by cation- and anion-exchange chromatography. N-TFA *n*-butyl esters.
[b] *n*-Butyl stearate as internal standard.
[c] Each value represents an independent analysis.

When drying the samples on the 100° sand bath with filtered gas, it is essential that the nitrogen gas is pure, and that the samples are taken just to dryness. Prolonged heating results in some breakdown of the samples.

A 50-molar excess or greater of TFAA/total amino acids should be used in the acylation medium, with the ratio of TFAA/CH_2Cl_2 being dependent on the sample concentration.

The chromatographic columns should be prepared and well conditioned as reported by GEHRKE *et al.*[13,15].

Performance blanks on all reagents should be made to check the purity of all reagents. It is especially important to check the purity of the TFAA to make certain it has not hydrolyzed on standing to TFA.

Iron must be carefully excluded during use of the rotary evaporator and magnetic stirring to obtain accurate and reproducible values for methionine. It is recommended that the samples be analyzed shortly after acylation to prevent any reduction in the chromatographic peak for methionine.

In some instances, especially with microgram samples, the TFA peak tails into the chromatogram, resulting in incorrect values for the first few amino acid peaks. To overcome this problem, hold the temperature program at the TFA peak, allow the TFA peak to be eluted, then resume the temperature program.

Direct on-column injection or the use of glass-lined injection ports eliminate

thermal decomposition of threonine and arginine which was observed when all-metal flask heaters were used.

CONCLUSIONS

It is concluded that quantitative GLC analyses of amino acids can be performed accurately and precisely on the most complex physiological substances if the samples are properly cleaned prior to derivatization and analysis.

Ion-exchange techniques were found to be well suited for cleanup of biological samples with regard to the removal of substances which interfere with the GLC analysis of the protein amino acids.

Also, quantitative analysis of amino acids by GLC can be made on biological samples containing microgram amounts of amino acids or protein. Good results can be obtained on samples containing 1 to 20 μg of total amino acids or protein, and by concentrating the final 100 μl of acylation solution to 20 μl, and injecting 8 μl, the amount of starting material can be reduced to about 50 to 100 ng of each amino acid and still achieve a semiquantitative analysis. With very careful techniques, ultra pure reagents and exclusion of all moisture, analyses can be made at the 1 to 10 ng level.

The applicability of GLC analysis of biological materials at the semimicro and micro levels was further demonstrated with the analysis of ribonuclease and lysozyme isolated from human milk. These analyses were made in joint investigations with Dr. SHAHANI, B. DALALY, and R. EITENMILLER of the University of Nebraska, Lincoln.

It is also concluded that the amino acid N-TFA *n*-butyl ester derivative possesses a distinct advantage over the N-TFA methyl esters in that the *n*-butyl derivatives are not lost to any appreciable extent on concentration of the final acylated sample. Sample loss of the methyl esters becomes even more critical when analyses are performed on microgram and submicrogram amounts of amino acids, where concentration of the derivatized sample is necessary.

Manuscripts recently published in the *Journal of Chromatography* by ROACH AND GEHRKE describe in detail a new approach to chromatography[15], and a "direct esterification" method for forming the amino acid *n*-butyl ester derivatives directly from the amino acids[25], thus eliminating the formation of the amino acid methyl esters and the interesterification reaction steps. These advancements greatly enhance the overall speed and simplicity of sample preparation and GLC analysis of the amino acids.

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