CHROM. 5263

A NANOGRAM AND PICOGRAM METHOD FOR AMINO ACID ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY*

ROBERT W. ZUMWALT**, KENNETH KUO*** AND CHARLES W. GEHRKE§
University of Missouri, Columbia, Mo. 65201 (U.S.A.)

(Received January 18th, 1971)

SUMMARY

A gas–liquid chromatographic method has been developed for the analysis of samples containing nanogram to picogram amounts of amino acids. The procedural details of the method are presented with the reaction conditions for conversion of 1 to 1000 ng of amino acids to their N-trifluoroacetyl *n*-butyl esters. The developed method was found to be essentially quantitative. Recoveries were greater than 80% when 5 ng of each amino acid were taken through the total method by comparison with diluted macro standard solutions.

Also, an injection port solvent vent-chromatographic system was invented which allows injection of the total derivatized samples (up to 100 μl) on a standard packed analytical column. This device prevents the solvent and excess acylating reagent from traversing the column and entering the detector, while allowing quantitative transport of the amino acid derivatives through the column. Samples containing seventeen amino acids at the 50 and 5 ng levels were taken through the total derivatization and chromatographic procedure, and then analyzed by GLC incorporating the solvent vent device.

To achieve higher sensitivities, the detection of the N-trifluoroacetyl and N-heptafluorobutyryl *n*-butyl esters of selected amino acids by ^{63}Ni electron capture was studied. The minimum detectible amounts of various amino acid derivatives were determined by demonstrating that 1 to 50 pg can be clearly observed by this method. Studies were made on the N-TFA *n*-butyl ester derivatives of eight amino acids, and on the N-HFB *n*-butyl esters of methionine and cysteine.

Analyses of the water extracts of the Returned Lunar Samples (Apollo flights 11 and 12) are presented with none of the common amino acids being detected above the background detection limit of *ca.* 4 to 5 ng/gram. However, several unidentified chromatographic peaks were observed, particularly in the Apollo 12 sample, which

* Contribution from Missouri Agricultural Experiment Station Journal Series No. 6157. Approved by the Director. Supported in part by grants from the National Aeronautics and Space Administration (NGR 26-004-011-S5), the National Science Foundation (GB 7182), and the Experiment Station Chemical Laboratories.

** National Science Foundation Predoctoral Fellow, University of Missouri.
*** Chemist, University of Missouri.
§ Professor and Supervisor of the Experiment Station Chemical Laboratories, and Research Associate in Space Sciences. Address to whom reprint requests should be sent.

were not present in the corresponding procedural blanks. These substances, which appear to be derivatizable, could either be indigenous to the sample, contamination, or the result of the unusual chromatographic effect reported earlier by these authors[39].

This analytical method now enables reliable analyses to be conducted in areas of research in which the amounts of amino acids present in the system are extremely small. The applications of this method should encompass a number of research areas, including cell component fractions from healthy and diseased tissue, metabolic studies, the study of highly active peptides present in trace amounts in bio-systems, as well as the mode of action of pesticides and pharmaceuticals. The developed technique has already been used to gain information on the Apollo 11 and 12 Returned Lunar Samples.

INTRODUCTION

As the result of extensive investigations in many areas, chromatography has become established as the preferred method for amino acid analysis. For quantitative determinations, ion-exchange and gas–liquid chromatography (GLC) are clearly the techniques of choice. The classical studies of MOORE *et al.*[1,2], HAMILTON[3], PIEZ AND MORRIS[4], and others have advanced the analysis of amino acids by ion-exchange chromatography to its present state of sophistication.

More recently, GLC techniques for the analysis of amino acids have been investigated due to the inherent advantages of sensitivity, speed, and versatility. Several different amino acid derivatives have been studied and evaluated by many investigators. Reviews by BLAU[5], WEINSTEIN[6], and MCBRIDE AND KLINGMAN[7] presented discussions on many amino acid derivatives, including derivatization methods and analytical applications.

In 1962, ZOMZELY *et al.*[8] studied the N-trifluoroacetyl (N-TFA) *n*-butyl esters as possible derivatives for the GLC determination of amino acids. In 1965, LAMKIN AND GEHRKE[9] reported that these amino acid derivatives were well suited for GLC analysis with regard to volatility and chromatographic properties. Other derivatives which have been investigated include the phenylthiohydantoin and methyl 2,4-dinitrophenyl esters[10,11], the N-TFA *n*-amyl esters[12], the N-acetyl *n*-amyl esters[13], and the N-trifluoroacetyl (N-TFA) methyl esters[14,15]. Much interest has also centered on the trimethylsilyl (TMS) derivatives introduced by RÜHLMANN AND GIESECKE[16], due to the possibility of a one-step derivatization procedure, whereas almost all other derivatives are formed by two or more reaction steps. Various silylation reagents have been studied, and some of these include hexamethyldisilazane and trimethylchlorosilane[16], trimethylsilyldiethylamine[17], trimethylsilyldimethylamine[18], and N-trimethylsilyl N-methylacetamide[19].

KLEBE *et al.*[20] used bis(trimethylsilyl)acetamide to obtain single chromatographic peaks for all of the protein amino acids with the exception of arginine, but were unable to separate the derivatives of glycine and alanine from the bis(trimethylsilyl)acetamide on an SE-30 column. The introduction of bis(trimethylsilyl)-trifluoroacetamide by STALLING *et al.*[21] has solved the problem of separation of TMS glycine and alanine from the reagents and reaction products. Further studies on the reaction and chromatographic conditions have been reported[22,23], with the develop-

ment of a single-step derivatization procedure, and a single-column separation of the TMS derivatives of the twenty protein amino acids[24,25].

Further investigations of the N-TFA *n*-butyl ester derivatives by GEHRKE and coworkers have resulted in the development of a GLC technique for quantitatively analyzing the twenty protein amino acids and many non-protein amino acids. These studies have included the determination of the reaction conditions for quantitative derivatization[26] and chromatographic studies[24,27]. A monograph by GEHRKE *et al.*[28] presents macro, semimicro, and micro methods, reagents, chromatographic requirements, and sample ion-exchange cleanup for quantitative GLC analysis of the protein amino acids. Further refinements with regard to sample preparation have been reported by ROACH *et al.*[29] and by ROACH AND GEHRKE[30] and include a "direct esterification" method for forming the amino acid *n*-butyl esters directly from the amino acids, and a method which greatly reduces the time required for protein hydrolysis. Recently, the development and evaluation of this GLC method for the analysis of amino acids in complex biological substances, specifically blood plasma and urine, has been reported by GEHRKE *et al.*[31,32].

As both classical ion-exchange chromatography and GLC methods have become more advanced, the capability to detect extremely small quantities of amino acids has become a reality. The investigations by HAMILTON *et al.*[33,34] and HARE *et al.*[35,36] have resulted in limits of detection well below the 100-ng level using the classical ion-exchange chromatographic technique.

Concurrently, due to the presence of the highly sensitive hydrogen flame and electron capture detection systems, GLC methods were considered to have great promise for the detection of amino acids at the nanogram level and below.

In 1968, GEHRKE *et al.*[28] reported on the flame-ionization detection of 6 ng of each of fourteen amino acids. Due to the limited amount of sample that could be injected on a packed analytical column, that study was conducted on samples containing 100 ng of each amino acid, but confirmed that nanogram quantities of amino acids could be derivatized, passed through the GLC column, and detected.

The purpose of this investigation was to develop a general and reliable method for the GLC analysis of amino acids at the nanogram level. The three major considerations were: (a) reproducible and quantitative derivatizations, (b) the invention of a GLC solvent vent-chromatographic device which would permit the injection of the total derivatized sample (up to 100 μl) on a standard packed analytical column, and (c) the detection of the amino acid derivatives when injected at the nanogram and picogram levels.

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 (C.W.G.) and scientists of the NASA-Ames Consortium of six principal and eleven co-investigators under the direction of Dr. CYRIL PONNAMPERUMA, senior scientist of the Ames Research Center, NASA, Moffett Field, Calif.

EXPERIMENTAL

General

Apparatus and equipment

The following gas chromatographs were used: a Packard Instrument Co. 7300

Series dual-column gas chromatograph, with both hydrogen flame and ^{63}Ni electron capture detectors, and equipped with a Honeywell Electronik 16 dual-pen strip chart recorder; a Varian Aerograph Model 2100 gas chromatograph with four-column oven bath, four hydrogen flame detectors, two dual differential electrometers, and equipped with a Varian Model 20 dual-pen recorder; a Bendix 2500 Series gas chromatograph with a four-column oven bath, two hydrogen flame detectors, and equipped with a Varian Model 20 dual-pen recorder.

The derivatization at the submicrogram levels was conducted in the pyrex glass micro reaction vials described in ref. 28 and available from Analytical BioChemistry Laboratories, P.O. Box 1097, Columbia, Mo. 65201. Teflon-lined screw caps were used to tightly close the vials during the esterification and acylation steps.

An ultrasonic cleaner (Branson Instruments, Inc., Stamford, Conn.) was used for mixing the samples after the addition of the derivatization reagents.

An IR heat lamp was used for evaporation of solvents and reagents from the samples in the micro-reaction vials.

The solvent vent-chromatographic device can be obtained from Analytical BioChemistry Laboratories, Box 1097, Columbia, Mo. 65201.

Hamilton syringes (100, 50, and 10 μl) or glass micropipets were used for the addition of the derivatization reagents to the samples.

A 100° constant-temperature sand-bath was used to heat the samples during esterification and acylation of the amino acids.

The apparatus used for the preparation of macro standard mixtures of amino acid derivatives (0.2 to 4 mg of each) was that described by ROACH AND GEHRKE[29].

Chromatographic columns

Ethylene glycol adipate (EGA) on Chromosorb W, 0.65 W/W%. The EGA column packing can be procured from Regis Chemical Company, 1101 N. Franklin Street, Chicago, Ill. 60610 under Code No. 201033; ethylene glycol adipate, "Stabilized Grade", from Analabs Inc., Hamden, Conn.; Chromosorb W, 80/100 mesh, acid-washed is a Johns-Manville product, and can be obtained from Applied Science, State College, Pa. or Fisher Scientific, St. Louis, Mo. Acetonitrile (anhydrous, "Nanograde") can be procured from Mallinckrodt Chemical Works, St. Louis, Mo. Adsorbent traps containing charcoal and molecular sieve 5A can be obtained from Guild Corporation, P.O. Box 217, Bethel Park,Pa. 15102, Regis Chemical Company, and Supelco, Inc., Bellefonte, Pa.

For the preparation of 25 g of the EGA column packing, 24.84 g of Chromosorb W were weighed into a 500-ml ridged round-bottom flask, then anhydrous "Nanograde" acetonitrile was added until the liquid level was *ca.* 1/8 in. above the Chromosorb W. Ten milliliters of a solution containing 16.25 mg/ml of EGA in anhydrous "Nanograde" acetonitrile were then added to the flask containing the Chromosorb W. The flask was then rotated on a rotary evaporator, slowly removing the solvent at room temperature under partial vacuum for *ca.* 45 minutes. When the Chromosorb was still slightly damp, the vacuum was increased and the flask immersed in a 60° water-bath with continued rotation until the solvent was completely removed. At this point, no Chromosorb W packing should adhere to the inner wall of the flask during rotation. At the end of this period, the dry, freely-flowing column packing was poured into clean, dry 1.5 m × 4 mm I.D. glass columns with gentle tapping. Dry

silanized glass wool plugs were then placed in each end of the column to hold the packing in place. Prior to analytical use, the column was placed in the gas chromatograph and conditioned at 220° with a carrier flow of *ca.* 50 ml/min of pure nitrogen. Analyses could be made after conditioning for 1 h when 0.5 to 1 μg of each amino acid were injected. Longer conditioning times (8 to 24 h at 220°) are required for analyses at lower concentrations. When not in use, the columns should be kept at 200° in the chromatograph with a carrier flow of 20 to 50 ml/min. If the columns must be removed from the instrument, the ends should be tightly closed during storage to exclude atmospheric moisture. The EGA columns must not be subjected at any one time to temperatures in excess of 225° for longer than 1 to 2 h.

Column for the separation of His, Arg, Trp, and Cys. The development of a mixed-phase column for the separation and quantitative analysis of histidine, arginine, tryptophan, and cystine is the subject of a separate paper now in preparation by these authors. The mixed-phase column gives effective resolution of these amino acids and eliminates the necessity of the "subtraction–computation" method reported earlier[37] by complete separation of histidine from phenylalanine and aspartic acid.

Comments on the EGA on acid-washed Chromosorb W column. Filters containing a high-grade charcoal (an efficient adsorbent for hydrocarbons), $CaSO_4$, and Linde 5A indicating molecular sieve for water should be placed in the nitrogen, hydrogen, and air lines to the gas chromatograph. The charcoal end of the filters is connected to the gas inlet side. The purity of the carrier gas is very important, especially when analyses are made at the submicrogram level. Properly prepared columns should last *ca.* two months and give the desired separation for seventeen amino acid derivatives, depending on the individual column and the types of samples injected. Signs of column degradation are: loss of the glycine–valine separation, loss of resolution in the methionine–hydroxyproline–phenylalanine region, and loss of separation for the ornithine–*n*-butyl stearate pair. Also, the TFA peak will be eluted later in the chromatogram as the column deteriorates.

In our previous publications, reference is made to the chromatographic separation of the amino acid derivatives[27,38]. The packing composed of stabilized grade EGA and acid-washed Chromosorb W (heated at 140° for 12 h) is an excellent one and gives effective separation of seventeen amino acids. In our recent studies on chromatographic separations of the amino acids, it was found that heating the Chromosorb W at 140° for 12 h was unnecessary. However, certain lots of Chromosorb W may still require a heat treatment for the removal of surface-adsorbed water as described in ref. 27.

Injection port solvent vent-chromatographic system

A major obstacle associated with the GLC analysis of extremely small quantities of biological samples or samples containing very low concentrations of amino acids has been the limited sample volume that could be injected into a packed analytical chromatographic column. In many instances, reduction of the final sample volume to 10 μl or less is impractical, due to problems of maintaining intact the derivatives of the biological compounds at extremely low concentrations. On removal of the solvent and excess derivatization reagents, many derivatized compounds become susceptible to hydrolysis by atmospheric moisture. Also, the various derivatives in the solution may exhibit different solubilities and separate as oils or crystals when

the sample volume is reduced to a few microliters, thereby making quantitative analyses very difficult.

For these reasons, a solvent vent-chromatographic system was invented which would fulfil the following criteria:

(1) Allow the injection of 100 μl or more of the derivatized sample on the chromatographic column, thereby eliminating the need for concentration of the derivatized sample.

(2) Prevent the large volume of injected solvent and acylating reagent from traversing the chromatographic column and entering the detector, while allowing essentially quantitative transport of the amino acid derivatives to the detector. Of particular importance is the exclusion of trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA) from the EGA column, which gives rise to a large TFA interference peak when analyzing at high sensitivities. The initial design of this injection port solvent vent-chromatographic system is presented in Fig. 1.

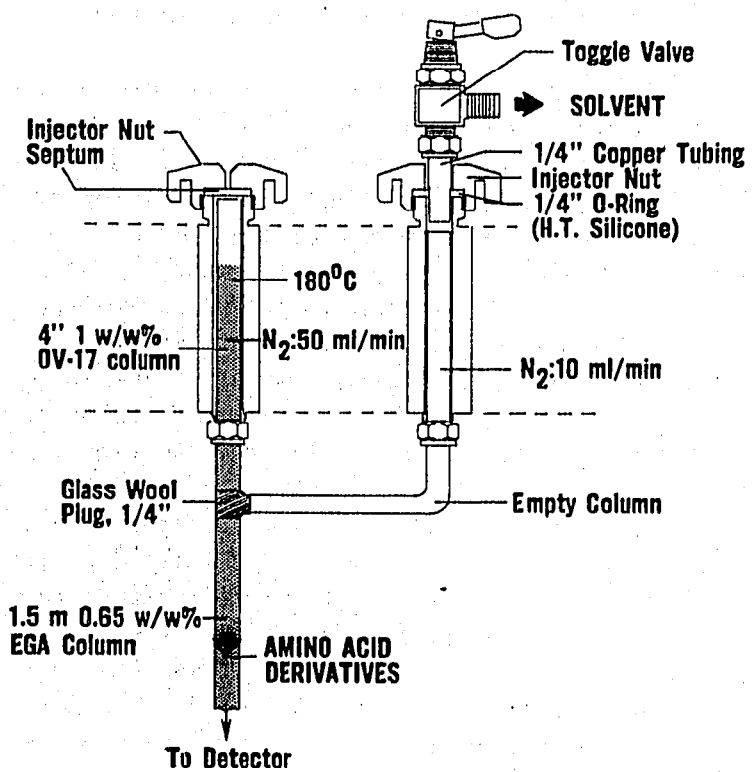

Fig. 1. Injection port solvent vent device.

Reagents

All of the amino acids were obtained from Mann Research Laboratories, Inc., New York, or Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure. A solution containing 0.1 mg/ml of each amino acid in 0.1 *N* HCl was used as a stock solution for preparing the amino acid derivatives. Amino acid reference standards, TAB(TM), from Regis Chemical Co., 1101 N. Franklin Street, Chicago, Ill. 60610, were used for confirmation and as standards.

n-Butanol and dichloromethane were redistilled from an all-glass system after

refluxing over calcium chloride. The *n*-butanol and dichloromethane were stored over Drierite in bottles with inverted ground glass tops. TFAA was obtained from Distillation Products Industries, Rochester, N.Y., and was an "Eastman Grade" chemical. The heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical Company, Rockford, Ill.

Redistilled diethyl ether was used as the solvent for the N-TFA and N-heptafluorobutyryl (N-HFB) *n*-butyl ester derivatives of the amino acids for electron capture studies.

An all-glass system was used for the production and purification of the HCl gas. The generator consisted of a 2000-ml reservoir containing *ca.* 100 ml of concentrated sulfuric acid. An attached 100-ml addition funnel was used for the slow addition of concentrated HCl to the reservoir. The resultant HCl gas was then passed through two sulfuric acid drying towers before bubbling into the *n*-butanol. The normality of the HCl in *n*-butanol was determined by weight and confirmed by titration.

Mixed phase column packing for His, Arg, Trp, and Cys is available from Analytical Biochemistry Laboratories, P.O. Box 1097, Columbia, Mo., Regis Chem. Co., Chicago, Ill. 1101, N. Franklin Street as Tabsorb-HAc, 100/120 mesh; and Supelco, Inc., Bellefonte, Pa.

Derivatization and chromatographic methods

The following procedure was developed and used for the derivatization and chromatographic analysis of extremely small amounts of amino acids as their N-TFA *n*-butyl esters.

(1) An aliquot of an aqueous (or weakly acidic) solution containing a mixture of amino acids (5 to 100 ng of each) was placed in a micro reaction vial, and evaporated just to dryness under an IR lamp. Samples of larger volume were placed in 5- to 10-ml beakers, evaporated to *ca.* 0.5 ml, then transferred to the vial with 0.1 *N* HCl. A black surface was used to support the samples, thus increasing the efficiency of the evaporative step. Clean watch glasses were suspended above the samples to aid in the exclusion of air-borne contamination. After all visible moisture had been removed from the reaction vials, the vials were tightly closed with the teflon-lined caps and allowed to cool. Then 50 μl of anhydrous dichloromethane were added, and evaporated under the IR lamp to azeotropically remove the last traces of moisture.

(2) For esterification of the amino acids to the *n*-butyl esters, 100 μl of *n*-butanol · 3 *N* HCl were added to the reaction vials. The vials were then tightly capped and placed in the ultrasonic bath just to the liquid level in the vial for *ca.* 30 sec. Water from the ultrasonic bath must be carefully excluded in this step. The exteriors of the vials were dried with tissue.

(3) The vials were then placed in a 100° sand-bath for 30 min. Only the lower portion of the vial containing the liquid was submerged in the sand, thus maintaining a liquid phase and allowing the sample to reflux. The samples were then removed from the sand-bath and allowed to cool.

(4) After the esterification step, the samples were evaporated just to dryness by removing the cap from the vials, and placing them under the IR lamp.

(5) The vials were then tightly capped, allowed to cool, then 50 μl of dichloromethane were added and evaporated under the IR lamp as in Step 1.

(6) The amino acid *n*-butyl esters were trifluoroacylated by the addition of

100 μl of TFAA–dichloromethane solution. The amount of TFAA present was varied from 0.5 to 10 μl per 100 μl solution. The vials were again tightly capped, sonicated for *ca.* 30 sec, and then heated in the 100° sand-bath for 10 to 20 min, depending on the wall thickness of the reaction vial used. For the analysis of arginine, tryptophan, and cystine, acylation at 150° for 5 to 10 min yields more quantitative results. After allowing the samples to cool to room temperature, analyses were made by GLC.

Some important observations of the derivatization and chromatography are presented in COMMENTS ON THE DERIVATIZATION METHOD.

Electron capture detection studies

The advent of the halogen-sensitive electron capture detector has aided greatly in the analysis for compounds possessing electronegative substituents. Therefore, studies were conducted to evaluate the ^{63}Ni detection of the N-TFA and N-HFB derivatives of the amino acid *n*-butyl esters. Standard solutions containing 1.0 mg of representative amino acids were derivatized and then made to a final volume of 10 ml. One-milliliter aliquots (100 μg) of the solutions were placed in separate 10-ml volumetric flasks, and the dichloromethane and acylating reagents which interfere with electron capture detection were evaporated with a stream of dry, purified nitrogen gas at room temperature. The derivatives were then redissolved in 10 ml (10 μg/ml) of freshly prepared absolute diethyl ether (distilled over metallic sodium). The solutions were thoroughly mixed, and appropriate aliquots and volumetric flasks were used to prepare additional samples at lower concentrations. The minimum detectable amounts of the amino acid derivatives were then determined at signal to noise ratios of 3:1 using electron capture detection. The analyses must be made immediately after derivatization and solution in ether.

RESULTS AND DISCUSSION

The N-TFA and N-HFB derivatives of the amino acid *n*-butyl esters were evaluated to determine the limit of detection at which these derivatives could be analyzed using a ^{63}Ni electron capture detector. Of primary interest was the comparison of these derivatives, which contain three and seven fluorine atoms per acyl group, respectively, with regard to detector response. The chromatographic properties of the N-TFA derivatives are well known, but more information on the N-HFB derivatives was desired.

Fig. 2 presents the chromatogram obtained on injection of a solution containing 3 ng of phenylalanine and 0.3 ng of tyrosine as the N-TFA *n*-butyl ester derivatives. The response of the tyrosine derivative is notably greater than that of phenylalanine, due to the additional trifluoroacetyl group. However, at these low levels of concentration, the tyrosine derivative was found to be much more susceptible to hydrolytic degradation than the phenylalanine derivative. This effect resulted in approximately equal minimum detectible amounts for these two amino acid derivatives.

The chromatograms obtained on analysis of the N-HFB *n*-butyl ester of methionine are presented in Fig. 3, showing the detection of both 33 and 3.3 pg injected of this derivative. Fig. 4 presents a similar study with the N-TFA derivative of hydroxyproline, showing that the detection of 10 and 2 pg of this derivative was

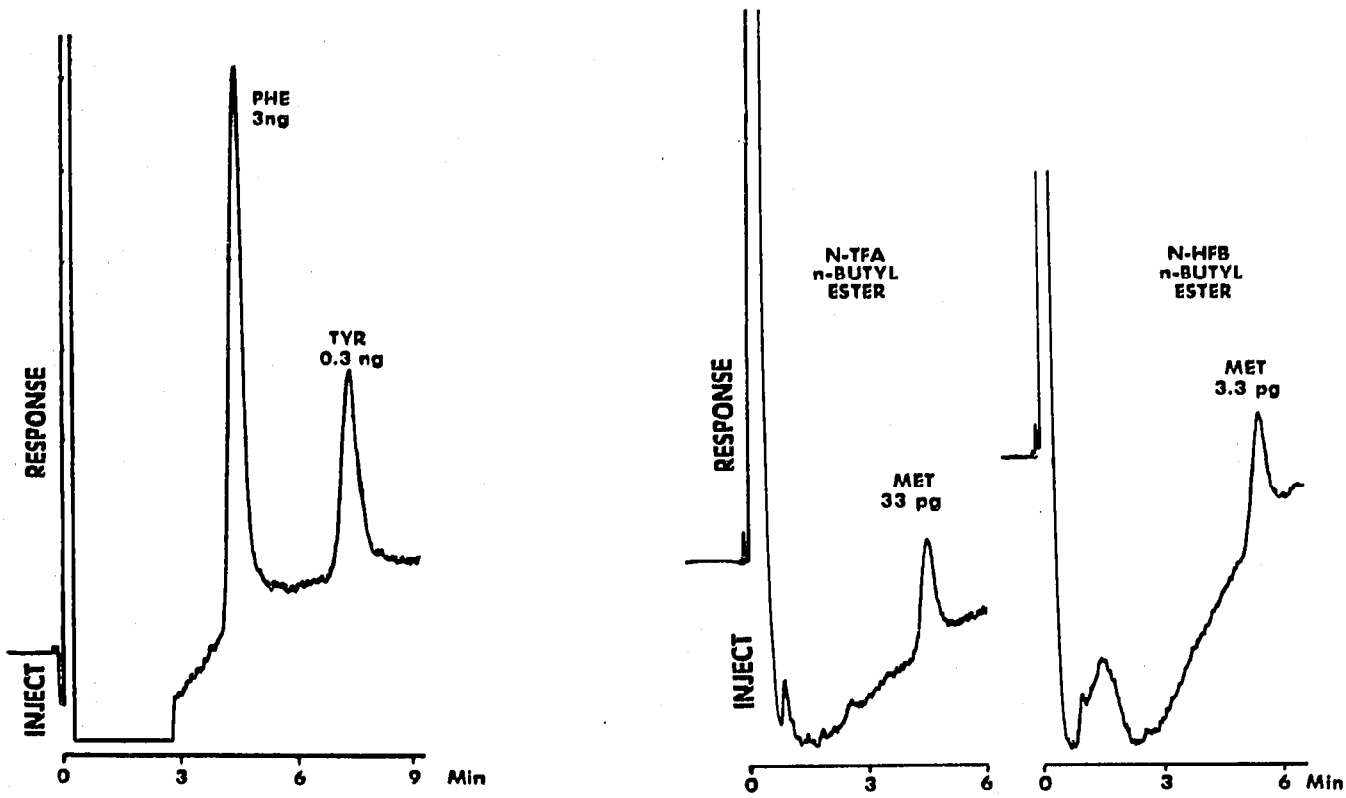

Fig. 2. GLC analysis of picogram amounts of the N-TFA *n*-butyl esters of phenylalanine and tyrosine. Column: 3.0 w/w % OV-101 on 80/100 mesh HP Chromosorb G, 1 m × 4 mm I.D. glass. Conditions: solvent, diethyl ether; detector voltage, 2.5 V d.c.; standing current, 4×10^{-9} A; detector temperature, 250°; column temperature (iso), 165°; attenuation, 1×10^{-10} a.f.s. Detection was carried out with a ^{63}Ni electron capture detector.

Fig. 3. GLC analysis of picogram amounts of the N-TFA and N-HFB *n*-butyl esters of methionine. Column, see the legend to Fig. 2. Column temperature (iso), 145°; attenuation, 1×10^{-10}, 3×10^{-11} a.f.s. For further conditions, see the legend to Fig. 2.

achieved. Further studies with other selected amino acids showed that the minimum detectable amounts (MDA) varied widely indeed, depending on the number of available sites on the amino acid that the fluorine-containing acyl group could be attached to. Table I gives the MDA of selected amino acids as the N-TFA derivatives. These values range from 2 to 55 pg of the amino acid derivatives injected. Under the experimental conditions used, the MDA of methionine and cysteine as the N-HFB *n*-butyl esters were 1 and 2 pg, respectively.

A major limitation of electron capture detection of amino acid derivatives is the difficulty of temperature programming. This limits the number of amino acids which may be eluted under a single set of chromatographic parameters. Flame-ionization detection, of course, allows temperature programming of the column oven, permitting analysis of essentially all the protein amino acids with a single sample injection. This feature becomes increasingly important when very small amounts of sample are available, and also with regard to analysis time.

Analyses of samples containing submicrogram quantities of seventeen protein amino acids were successfully conducted, using the derivatization procedure and the

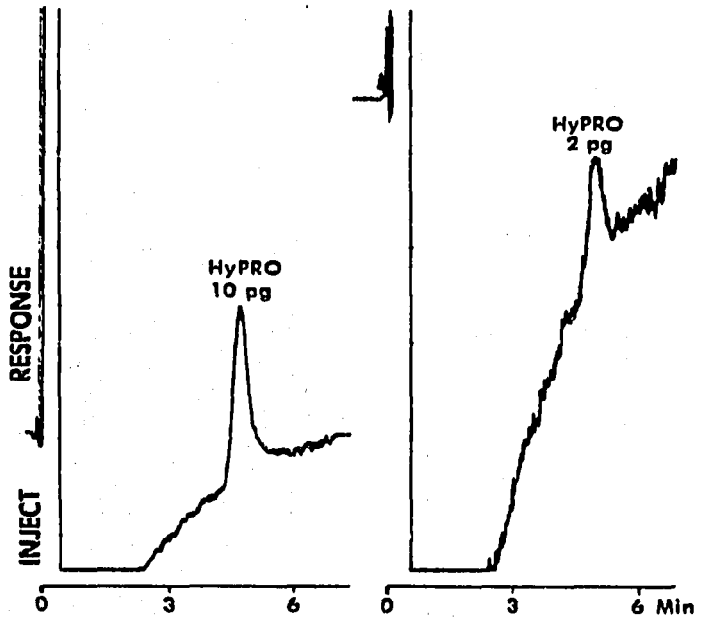

Fig. 4. GLC analysis of picogram amounts of the N-TFA *n*-butyl esters of hydroxyproline. Column, see the legend to Fig. 2. Column temperature (iso), 140°; attenuation, 1×10^{-10}, 3×10^{-11} a.f.s. For further conditions, see the legend to Fig. 2.

injection port solvent vent-chromatographic system described under EXPERIMENTAL. Fig. 5 shows the chromatogram obtained after an aqueous solution containing 50 ng of each amino acid had been taken through the entire derivatization and GLC procedure. The limit of analysis was further extended with the successful derivatization and GLC analysis of samples containing only 5 ng of each amino acid, as seen in Fig. 6.

TABLE I

MINIMUM DETECTABLE AMOUNTS OF SELECTED AMINO ACIDS[a]

Amino acid	*MDA*[b] *(picograms injected)*	
	N-TFA[c]	*N-HFB*[c]
Alanine	50	
Serine	5	
Methionine	10	1
Cysteine	5	2
Proline	50	
Hydroxyproline	2	
Phenylalanine	55	
Tyrosine	45	

[a] ^{63}Ni electron capture detection.
[b] Based on a signal to noise ratio of 3:1.
[c] Amino acid *n*-butyl esters in diethyl ether.

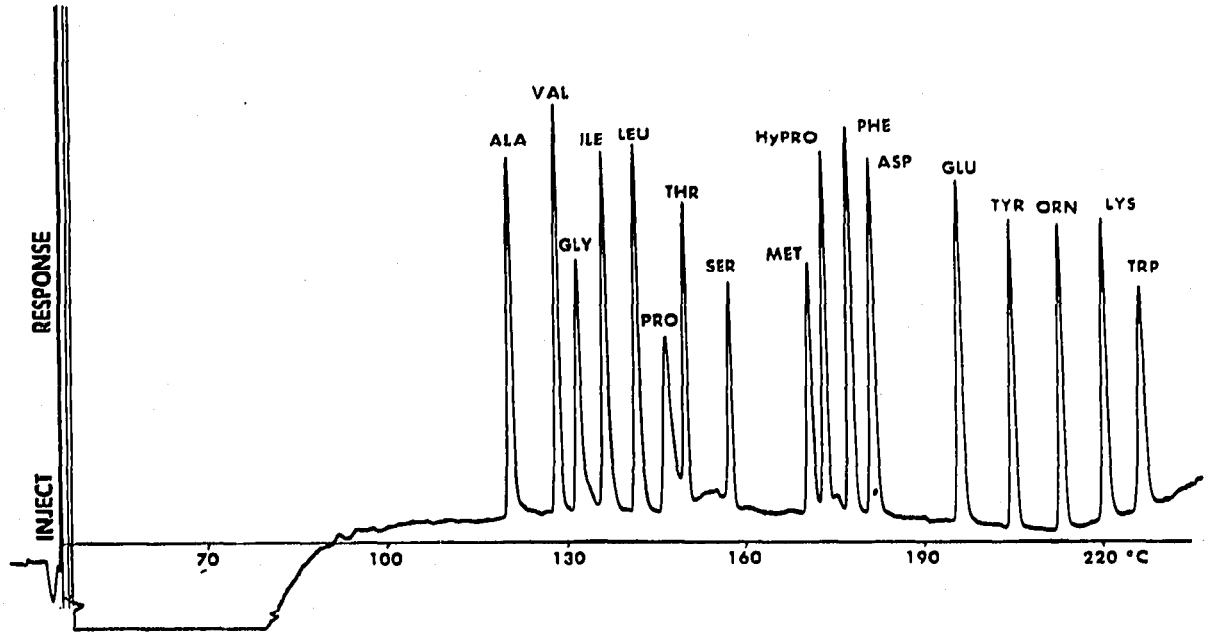

Fig. 5. Derivatization and GLC analysis of 50 ng of each amino acid. Column: 0.65 w/w % EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Pre-column: 1.0 w/w % OV-17 on 80/100 mesh HP Chromosorb G, 4 in. × 4 mm. Esterification was carried out with 25 μl *n*-butanol·3 *N* HCl at 100° for 70 min; acylation with 25 μl TFAA–dichloromethane (1:9) at 100° for 20 min. Conditions: sample injected, 25 μl; solvent vent time, 30 sec; injection port temperature, 150°; initial temperature, 70°; initial hold, 4 min; program rate, 6°/min; attenuation, 4×10^{-11} a.f.s.

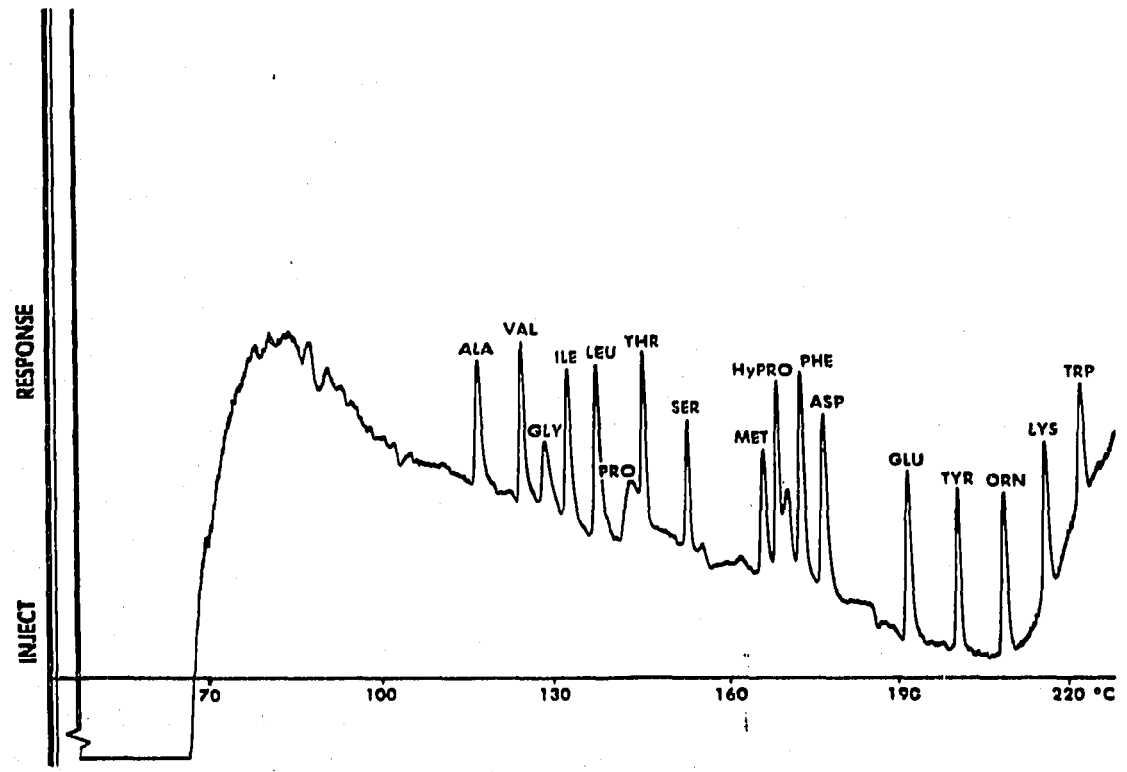

Fig. 6. Derivatization and GLC analysis of 5 ng of each amino acid. Column and pre-column, see the legend to Fig. 5. Derivatization and acetylation were carried out in the same way as described in the legend to Fig. 5. Attenuation, 8×10^{-12} a.f.s. For further conditions, see the legend to Fig. 5.

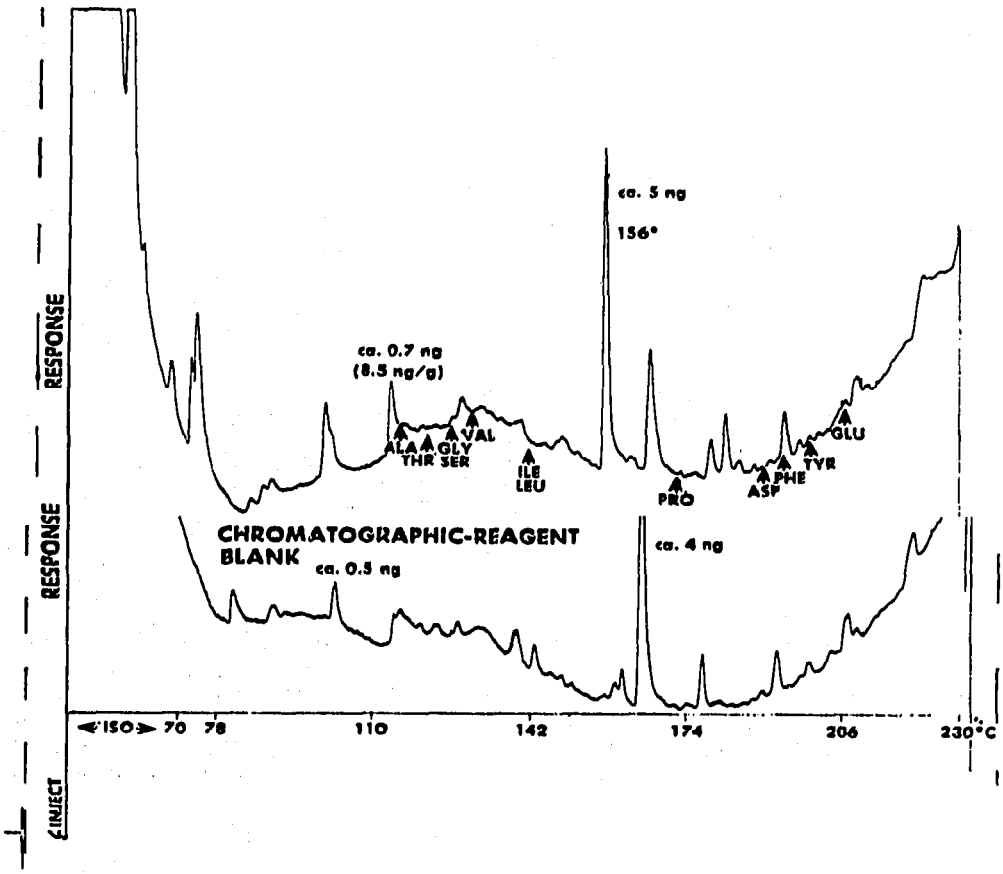

Fig. 7. GLC chromatogram obtained on analysis of the water extract of Lunar material returned by Apollo flight 11. Column: 1.0 w/w % OV-17 on 80/100 mesh HP Chromosorb G, 2.5 m × 2 mm I.D. glass. Sample: 1.1 g of Lunar fines (3.3 ml); the samples were refluxed with triply distilled water for 17 h. Derivatization was carried out with 100 μl *n*-butanol·3 *N* HCl; acetylation with 100 μl TFAA–dichloromethane (0.5:100). Conditions: final volume, 60 μl; sample injected, 5 μl; injection port temperature, 280°; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; final temperature, 232°; attenuation, 4 × 10^{-12} a.f.s.

ANALYSIS OF APOLLO 11 AND 12 SAMPLES

The GLC technique was used in the search for amino acids in the Returned Lunar Samples. Figs. 7 and 8 present the chromatograms obtained on analysis of the water extracts of Lunar material returned by Apollo flights 11 and 12, respectively. No amino acids could be identified above the background detection level of *ca.* 4 to 5 ng/g of each amino acid. However, analysis of the Lunar samples resulted in numerous chromatographic peaks which were apparently not present in the corresponding blanks, as seen in Figs. 7 and 8. This difference is particularly evident on observation of the results obtained from the Apollo 12 studies (Fig. 8). Although these peaks did not correspond to any of the protein amino acids, no further identification of these materials was made. These substances could be either indigenous to the sample, contamination, or the result of the unusual chromatographic effect reported earlier by these authors[39].

During these studies, scrupulous monitoring of possible sources of contamination was always necessary. To determine the level of contamination that could be

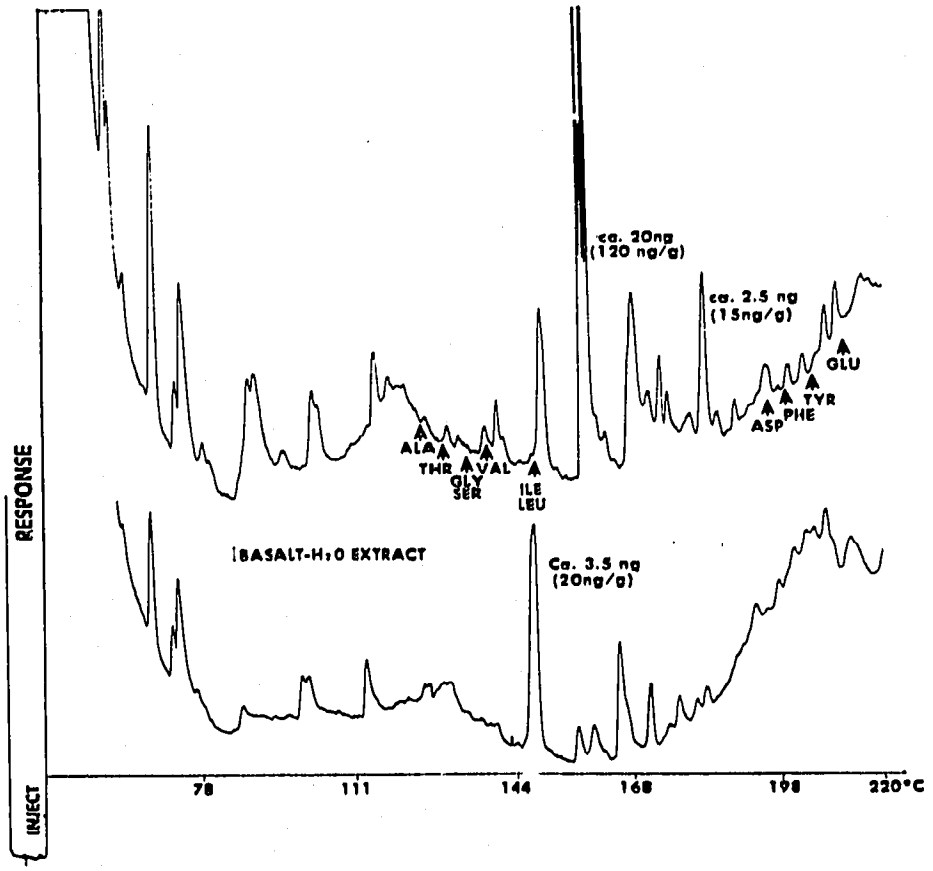

Fig. 8. GLC chromatogram obtained on analysis of the water extract of Lunar material returned by Apollo flight 12. Column, see the legend to Fig. 7. Sample: 1.2 g of Lunar fines (6 ml); the samples were refluxed with triply distilled water for 13 h. Derivatization and acetylation were carried out as described in the legend to Fig. 7. Final volume, 30 μl; final temperature, 220°. For further conditions, see the legend to Fig. 7.

introduced by a fingerprint, the following experiment was conducted: The authors carefully washed their hands, thoroughly rinsed them with cold distilled water, and then placed four fingerprints by gently touching the wall of a 15-ml beaker. Five milliliters of 0.1 *N* HCl were added to the beaker, carefully washing the sides of the beaker. The aqueous solution was then evaporated to *ca.* 0.5 ml under an IR lamp, transferred to a micro reaction vial, and then derivatized to the N-TFA *n*-butyl esters with a final volume of 100 μl. A companion blank was taken through the same procedure.

The chromatogram obtained on GLC analysis of the fingerprint sample is presented in Fig. 9. About 1 μg of total amino acids were found per fingerprint. The analysis of the companion blank resulted in no significant chromatographic peaks when analyzed at a sensitivity four times greater. HAMILTON[33] had earlier investigated contamination from amino acids resulting from thumbprints by classical ion-exchange chromatography and reported that the detection of each of seventeen amino acids ranged from *ca.* 500 to 20 ng per dry thumbprint. Serine and glycine were the most abundant amino acids resulting from wet thumbprints with *ca.* 10 and 7 μg being reported, respectively.

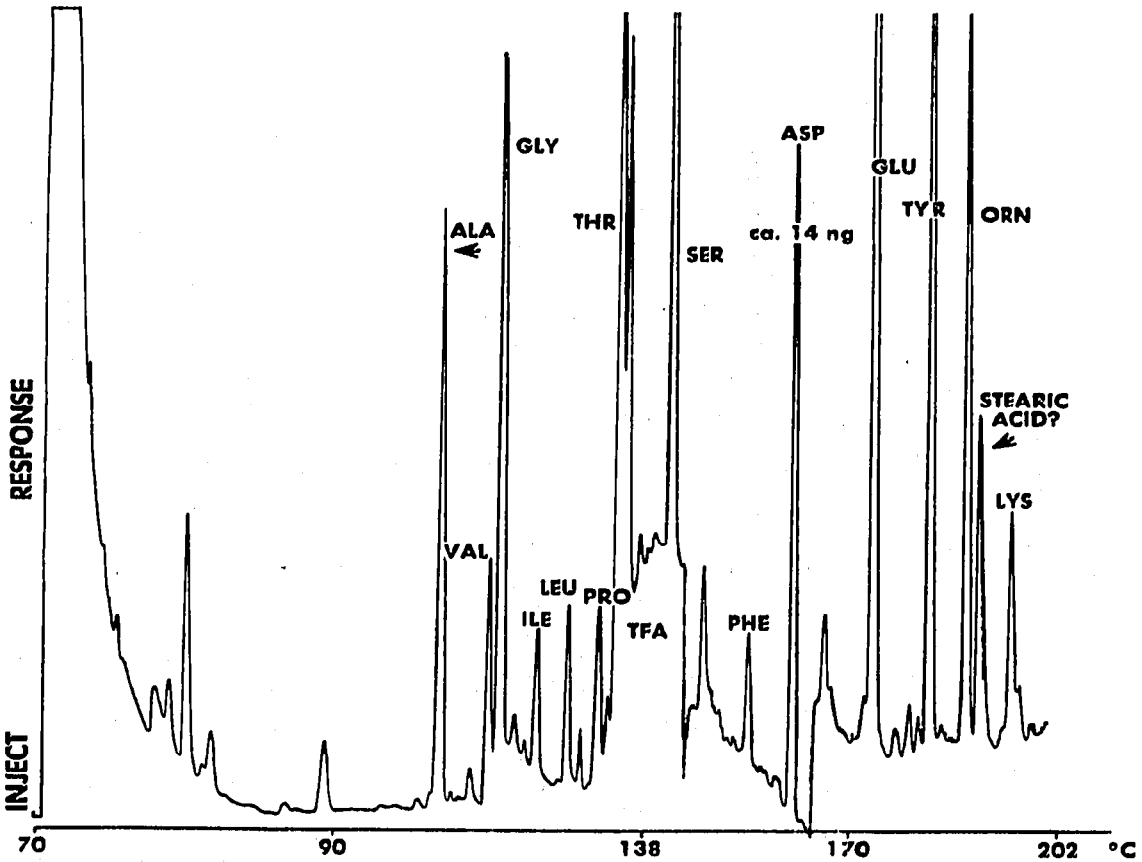

Fig. 9. Chromatogram obtained on GLC analysis of fingerprint samples of N-TFA *n*-butyl esters of amino acids. Column: 0.325 w/w % stabilized grade EGA on 80/100 mesh AW Chromosorb G, 1.5 m × 2 mm I.D. glass. Conditions: sample, four fingerprints; sample volume, 100 μl; sample injected, 4 μl; initial temperature, 70°; initial hold, 6 min; program rate, 4°/min; attenuation, 8×10^{-10} a.f.s. Approximate amounts of amino acids per fingerprint in nanograms: Ala, 80; Val, 30; Gly, 125; Ile, 25; Leu, 25; Pro, 25; Thr, 125; Ser, 120; Phe, 20; Asp, 90; Glu, 160; Tyr, 140; Orn, 120; Lys, 30 (totalling 1115 ng).

COMMENTS ON THE DERIVATIZATION METHOD

(1) The purity of all reagents and the cleanliness of all glassware used throughout the procedure is of obvious importance when analyzing at these low levels of amino acid concentration.

The distillation of the required reagents, described in *Reagents*, must be carefully conducted. To monitor the purity of the redistilled *n*-butanol and dichloromethane, 10-ml aliquots of the redistilled products were evaporated to *ca.* 0.25 ml, and then analyzed by GLC under the chromatographic conditions to be used for analysis of the derivatized sample. The Drierite or molecular sieve over which the distilled solvents are stored should be rinsed with portions of the anhydrous reagent prior to use. All reagent bottles must be kept tightly closed and covered to exclude atmospheric moisture and air-borne dust particles.

(2) Effective techniques for obtaining clean glassware for use in the derivatization procedure include soaking the glassware in hot chromic acid cleaning solution for 4 to 8 h, followed by exhaustive rinsing with triply distilled water. Alternatively,

in addition to thorough cleaning, the necessary glassware may be placed in a furnace and heated overnight at 500° to remove contaminating organics, and final rinsing with the reagents to be used.

(3) Moisture must be carefully excluded during the entire derivatization procedure. The *n*-butanol, HCl gas, and dichloromethane must be anhydrous, and the TFAA should not contain significant amounts of TFA.

(4) It is essential that reagent blanks be analyzed particularly often, as amino acid contamination can be easily introduced from a variety of sources, especially from fingerprints.

(5) Performance standards must be analyzed to ensure the integrity of the derivatization reagents. The concentration of the amino acids in the performance standard should be similar to the amino acid concentrations in the samples to be analyzed.

CONCLUSIONS

Refinements have been made in the chemical derivatization and chromatography for the determination of the protein amino acids as the N-TFA *n*-butyl esters. The amino acids were reproducibly determined at the nanogram (10^{-9} g) and picogram (10^{-12} g) level. Recoveries of greater than 80% were achieved when 5 ng of each amino acid were taken through the total derivatization and chromatographic method, thus showing that losses due to adsorptive and substrate–derivative interactions were minimal. Of importance is the invention by the authors of a "solvent vent-chromatographic device" which greatly simplifies the analysis of nanogram and picogram amounts. In many instances, particularly in biological studies, the derivatized compounds of interest are subject to degradation by atmospheric moisture—oxygen—or possess differing solubilities in the solvent used, thereby making concentration of the final derivatized sample hazardous. This "device" eliminates the need for sample concentration and the large solvent peak with resultant "tailing" into the eluted peaks on the chromatogram. Of particular importance when chromatographing the amino acid derivatives with this venting device is the complete exclusion of the TFA reagent peak from the EGA column. When operating at high instrumental sensitivities as was required in the "search for organics" in the Returned Lunar Samples (Apollo flights 11 and 12), the TFA and TFAA reagents gave rise to a large interference in the chromatogram. Further, this "device" lowers the demands that must be made on the instrumentation, by allowing the injection of a broad range of sample volumes (0.1 to 200 μl). This "solvent vent-chromatographic device" should be useful in a wide range of applications of GLC and GLC–mass spectrometry studies. To achieve an even higher degree of sensitivity, the N-TFA and N-HFB *n*-butyl esters were determined using electron capture detection. These derivatives were clearly detectible at levels of 1 to 50 pg of each amino acid. Also, as noted in Fig. 6, contamination can be kept to a minimum. Although amino acids are notorious in this regard, the level of amino acid contamination encountered throughout derivatization and GLC chromatographic analysis can be kept below the 1-ng level in the typical laboratory surroundings. This is primarily due to the use of purified organic reagents and the small quantities required for chemical derivatization.

This method now permits reliable studies to be conducted in areas in which the

expected content of the compounds of interest is extremely small. The broad spectrum of applications for this technique has already included the search for amino acids in the Returned Lunar Samples, and would range from studies at the cellular level, investigations on the molecular basis of disease, to the analysis for trace amounts of environmental pollutants.

REFERENCES

1 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 192 (1951) 663.
2 S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
3 P. B. HAMILTON, *Anal. Chem.*, 35 (1963) 2055.
4 K. A. PIEZ AND L. MORRIS, *Anal. Chem.*, 1 (1960) 187.
5 K. BLAU, *Biomed. Appl. Gas Chromatogr.*, 2 (1968) 1.
6 B. WEINSTEIN, *Methods Biochem. Anal.*, 14 (1968) 1.
7 W. J. MCBRIDE, JR. AND J. D. KLINGMAN, *Lect. Gas Chromatogr.*, 2 (1968) 1.
8 C. ZOMZELY, G. MARCO AND E. EMERY, *Anal. Chem.*, 34 (1962) 1414.
9 W. M. LAMKIN AND C. W. GEHRKE, *Anal. Chem.*, 37 (1965) 383.
10 J. J. PISANO, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochem. Biophys. Res. Commun.*, 7 (1962) 82.
11 J. J. PISANO AND T. J. BRONZERT, *J. Biol. Chem.*, 244 (1969) 20, 3397.
12 A. DARBRE AND K. BLAU, *Biochem. J.*, 88 (1963) 8.
13 D. E. JOHNSON, S. J. SCOTT AND A. MEISTER, *Anal. Chem.*, 33 (1961) 669.
14 H. A. SAROFF AND A. KARMEN, *Anal. Biochem.*, 1 (1960) 344.
15 P. A. CRUICKSHANK AND J. C. SHEEHAN, *Anal. Chem.*, 36 (1964) 1191.
16 K. RÜHLMANN AND W. GIESECKE, *Angew. Chem.*, 73 (1961) 113.
17 E. D. SMITH AND H. SHEPPARD, JR., *Nature*, 208 (1965) 878.
18 *Chromatography/Lipids* (Supelco, Inc., Bellefonte, Pa.), 1 (1967) 3.
19 L. BIRKOFER AND M. DONIKE, *J. Chromatog.*, 26 (1967) 270.
20 J. KLEBE, H. FINKBEINER AND D. WHITE, *J. Amer. Chem. Soc.*, 88 (1966) 2290.
21 D. L. STALLING, C. W. GEHRKE AND R. W. ZUMWALT, *Biochem. Biophys. Res. Commun.*, 21 (1968) 616.
22 C. W. GEHRKE, H. NAKAMOTO AND R. W. ZUMWALT, *J. Chromatogr.*, 45 (1969) 24.
23 H. NAKAMOTO, *Masters' Thesis*, University of Missouri, June, 1969.
24 C. W. GEHRKE, R. W. ZUMWALT AND K. KUO, *J. Chromatogr.*, 57 (1971) 209.
25 K. LEIMER, *Masters' Thesis*, University of Missouri, June, 1971.
26 C. W. GEHRKE AND D. L. STALLING, *Separ. Sci.*, 2 (1967) 101.
27 D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 43 (1969) 303.
28 C. W. GEHRKE, D. ROACH, R. W. ZUMWALT, D. L. STALLING AND L. L. WALL, *Quantitative Gas–Liquid Chromatography of Amino Acids in Proteins and Biological Substances*, Analytical BioChemistry Laboratories, Columbia, Mo., 1968.
29 D. ROACH, C. W. GEHRKE AND R. W. ZUMWALT, *J. Chromatogr.*, 44 (1969) 269.
30 D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 52 (1970) 393.
31 R. W. ZUMWALT, D. ROACH AND C. W. GEHRKE, *J. Chromatogr.*, 53 (1970) 2, 171.
32 R. W. ZUMWALT, K. KUO AND C. W. GEHRKE, *J. Chromatogr.*, 55 (1971) 267.
33 P. B. HAMILTON, *Nature*, 205 (1965) 284.
34 B. NAGY, C. M. DREW, P. B. HAMILTON, V. W. MODZELESKI, SISTER M. E. MURPHY, W. M. SCOTT, H. C. UREY AND M. YOUNG, *Science*, 167 (1970) 3918, 770.
35 P. E. HARE, *Fed. Proc.*, 25 (1966) 709.
36 S. W. FOX, K. HARADA, P. E. HARE, G. HINSCH AND G. MUELLER, *Science*, 167 (1970) 3918.
37 D. ROACH, C. W. GEHRKE AND R. W. ZUMWALT, *J. Chromatogr.*, 43 (1969) 311.
38 C. W. GEHRKE, R. W. ZUMWALT AND L. L. WALL, *J. Chromatogr.*, 37 (1968) 398.
39 C. W. GEHRKE, R. W. ZUMWALT, W. A. AUE, D. L. STALLING AND J. J. RASH, *J. Chromatogr.*, 54 (1971) 169.