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AMINO ACID ANALYSIS

HYDROLYSIS, ION-EXCHANGE CLEANUP, DERIVATIZATION, AND QUANTITATION BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

Sample preparation techniques are as vital in amino acid analysis as the methods by which the amino acids are measured.

Experiments were conducted to obtain a rapid, accurate, and precise procedure for protein hydrolysis and sample cleanup with subsequent gas–liquid chromatographic analysis. The use of ultrasonication to remove dissolved air while pulling a vacuum on the sample solution prior to hydrolysis assured a good recovery for methionine and cystine. These techniques combined with a 4-h hydrolysis at 145° using 6 *N* HCl gave results in good agreement with the hydrolysis conditions of 18–24 h at 110°.

Physiological fluids for free amino acids were prepared by precipitating the protein with saturated picric acid followed by cation-exchange clean-up. Gas–liquid chromatographic analysis of small samples was improved by the use of a Sol-Vent device. This enables one to inject large volumes of the derivatized sample (100 μl) onto the analytical column, eliminate the tailing effects of the solvent and trifluoroacetic acid while still retaining the amino acids on the analytical column.

The techniques for sample preparation and chromatographic analysis presented in this paper provide the chemist with valuable tools for the analysis of amino acids in biological samples by gas–liquid chromatography.

INTRODUCTION

The hydrolysis of proteins is extremely important in relation to amino acid analysis. Some amino acids are easily destroyed or altered while the hydrolysis of others is incomplete. The most common procedure in use today is hydrolysis with 6 *N*

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HCl for 18–24 h at 110°. This procedure is described by MacPherson[1] and by Moore and Stein[2]. The most serious problem with this procedure is the partial loss of some amino acids. Tryptophan is totally destroyed while threonine, serine, and tyrosine are partially destroyed. Methionine and cystine also are either partially destroyed or oxidized to methionine sulfone and cysteic acid. Other amino acids such as valine, isoleucine, and leucine are incompletely hydrolyzed during the 24-h period and must be hydrolyzed longer to obtain complete recovery. In 1970, Roach and Gehrke[3] reported on a hydrolysis procedure using 6 *N* HCl at 145° for a minimum of 4 h. They obtained results in good agreement with the standard procedure of 18–24 h at 110°. In 1973, Gehrke and Takeda[4] reported on a hydrolysis procedure using 6 *N* HCl with 5% thioglycolic acid at 110° for 20 h. With this method they were able to determine tryptophan in pure proteins; however, slightly decreased values for arginine and cystine were obtained. Other methods of hydrolysis have been tried such as alkaline or enzymatic hydrolysis. These procedures, however, result in partial destruction of some amino acids or in incomplete hydrolysis. One procedure developed by Liu and Chang[5] using *p*-toluenesulfonic acid and a protective reagent, 3-(2-aminoethyl)indole, does show promise for the hydrolysis of proteins for all amino acids. However, up to 72 h of hydrolysis time is needed for certain amino acids and when the carbohydrate content becomes high, the procedure is not applicable to tryptophan hydrolysis.

The use of gas–liquid chromatography (GLC) has become a very important method for the analysis of amino acids. Several types of derivatives have been used but the most reliable and common are the N-trifluoroacetyl (N-TFA) *n*-butyl esters developed by Gehrke and co-workers[6–10]. Excellent precision and accuracy have been demonstrated. Zumwalt, Kuo and Gehrke[9,10] have also reported on the use of an injection port Sol-Vent device, which allows injection of large amounts of samples. This results in greater sensitivity, accuracy, and precision, especially for very small samples.

The purpose of this study was to develop a sample preparation method for amino acid analysis of biological samples that is rapid, precise, and accurate. This along with the subsequent analysis of the amino acids by GLC would provide the chemist with a valuable tool for the quantitation of amino acids.

EXPERIMENTAL

Apparatus and supplies

A Varian Aerograph Model 2100 gas chromatograph with a four-column oven, two flame ionization detectors, dual differential electrometers and equipped with a Varian Model 20 recorder was used. Infotronics CRS-104 and 110A digital integrators were used for determining peak areas. Adsorbent trap filters containing molecular sieve 5A, charcoal, and drierite for the hydrogen, air, and nitrogen lines were obtained from Guild Corp. (Bethel Park, Pa., U.S.A.), Regis Chem. Co. (Morton Grove, Ill., U.S.A.) and Supelco (Bellefonte, Pa., U.S.A.).

An ultrasonic cleaner was used to mix the samples after the addition of reagents; ultrasonic bath, cat. No. 42-37-11 Balsonic I cleaner, from Bausch & Lomb, Rochester, N.Y., U.S.A.

A Sol-Vent system (Analytical BioChemistry Lab., Columbia, Mo., U.S.A.) was used for the injection of large amounts of derivatized sample onto the analytical column (patent applied for).

Constant-temperature oil-baths set at 100° and 150 ± 2° were used for esterification and acylation, respectively.

Whitey valve (a needle valve) can be obtained from Whitey Co., Oakland, Calif., U.S.A.; catalog No. ORS2.

A convection oven maintained at either 145° or 110 ± 2° was used for hydrolysis of samples.

Glass drying tube, inverted form with S 24/40 inner joint at one end (catalog No. 9-222) and 125-ml flask S 24/40, short neck, flat bottom (catalog No. 9-559A) were from Fisher Scientific, St. Louis, Mo., U.S.A.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser (all glass and PTFE) (CaLab Equipment Co., Oakland, Calif., U.S.A.) and a Model 1400 Welch Duo-Seal vacuum pump (Fisher Scientific).

Ion-exchange columns No. 2726-QB4 were obtained from Arthur H. Thomas Co. for the cation-exchange cleanup of samples. These were 15 × 150 mm and contained porous glass frits.

Morton flasks (a ribbed round-bottom S flask) were purchased from Scientific Glass Blowing Co., Houston, Tex., U.S.A.; catalog No. SGB-13310.

Pyrex 200 × 25 mm glass screw-top culture tubes (Corning No. 9826) with PTFE-lined caps were used as containers for hydrolysis of the protein samples. Pyrex 16 × 75 mm glass screw-top culture tubes (Corning No. 9826) with PTFE-lined caps were used as reaction vessels for the acylation reactions.

Micro reaction vials were obtained from Analytical BioChemistry Laboratories. PTFE-lined screw-caps were used to close the vials.

Reagents and materials

All amino acids used as standards were obtained from Mann Research Labs., New York, N.Y., U.S.A. or from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., and were chromatographically pure. Ribonuclease (bovine pancreas) five times crystallized was obtained from Mann Research Labs. High-lysine corn, fish meal, and blood plasma were obtained from Analytical BioChemistry Labs. Cation-exchange resin (Amberlite CG-120, 100–200 mesh) was obtained from Mallinckrodt, St. Louis, Mo., U.S.A. and was prepared as described under *Preparation of cation-exchange resin*.

Other reference compounds and accessories for amino acid analysis were those given on pp. 30 and 31 of the catalog of Regis Chemical Co.

Constant-boiling 6 *N* HCl was prepared by the distillation of a solution of concentrated HCl and doubly distilled water (conc. HCl–distilled water, 9:11, v/v).

n-Butanol was ACS reagent grade from Fisher Scientific. Methylene chloride was "nanograde" quality from Mallinckrodt. Trifluoroacetic anhydride (TFAA) was obtained from Distillation Products Industries, Rochester, N.Y., U.S.A. and was an "Eastman Grade" chemical. The internal standard *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (tranexamic acid) was obtained from Aldrich, Milwaukee, Wisc., U.S.A., No. S42955-4. Pure anhydrous HCl gas was generated by the slow addition of reagent-grade HCl into concentrated H_2SO_4. The HCl gas was passed through two drying towers containing concentrated H_2SO_4, then slowly bubbled into *n*-butanol until 3 *N* in HCl. The normality was checked by titration with standardized base. The GLC packings, 0.65% EGA on Chromosorb W AW, 80–100 mesh, and 2% OV-17 +

1% OV-210 on Gas-Chrom Q, 100–120 mesh, were obtained from Analytical Bio-Chemistry Labs. and were prepared as described in the next section.

Preparation of Chromatographic Column Packings

Packing I. Stabilized ethylene glycol adipate (EGA) on Chromosorb W, 0.65% (w/w)

Materials. Ethylene glycol adipate, stabilized grade (Analabs, Hamden, Conn., U.S.A.); Chromosorb W AW, 80–100 mesh (Johns-Manville product obtained from Applied Science Labs., State College, Pa., U.S.A.); acetonitrile anhydrous "nanograde" (Mallinckrodt).

Preparation of Packing I. For preparation of 30 g of Packing I, 29.805 g of Chromosorb W AW are weighed into a 500 ml Morton flask, then anhydrous "nanograde" acetonitrile is added until the liquid level is *ca.* 1/8 in. above the Chromosorb W.

Into a clean 100-ml beaker is weighed 0.195 g of EGA, then *ca.* 20 to 25 ml of anhydrous "nanograde" acetonitrile are added and the EGA dissolved. Use of an ultrasonic bath aids in dissolving the EGA. The EGA solution is then added to a Morton flask containing the Chromosorb W and the flask is placed on a rotary evaporator (all-glass or PTFE). The solvent is slowly removed under partial vacuum at room temperature for *ca.* 1 h. When the Chromosorb is still damp, but does not adhere to the side of the flask, remove the flask from the evaporator and wash the inside walls of the flask with a few milliliters of anhydrous "nanograde" acetonitrile. Place the flask on the evaporator and continue to remove the solvent under partial vacuum. When the packing is only slightly damp, the vacuum is increased and the flask immersed in a 60 water-bath with continued rotation until the solvent is completely removed. At this point, no EGA packing should adhere to the inner wall of the flask.

To prepare the chromatographic column, the packing is poured into clean, dry 1.5 m × 4 mm I.D. glass columns with gentle tapping. Silanized glass-wool plugs are placed in each end to hold the packing in place.

Packing II. 2.0% (w/w) OV-17 + 1.0% (w/w) OV-210 on Gas-Chrom Q

Materials. OV-17 (Applied Science Labs.); OV-210 (Applied Science Labs.); Gas-Chrom Q, 100–120 mesh (Applied Science Labs.); acetone, anhydrous "nanograde" (Mallinckrodt).

Preparation of Packing II. For preparation of 30 g of Packing II, 29.1 g of Gas-Chrom Q are weighed into a 500-ml Morton flask, then anhydrous "nanograde" acetone is added until the liquid level is *ca.* 1/8 in. above the Gas-Chrom Q. Into clean, dry 100-ml beakers are weighed 0.600 g of OV-17 and 0.300 g of OV-210, then *ca.* 20 to 25 ml of anhydrous "nanograde" acetone are added and the liquid phases dissolved. Each liquid phase solution is then added to the Morton flask and the solvent is removed in the same manner as described above for preparation of Packing I. The dried column packing is placed in 1.5 m × 4 mm I.D. glass columns and plugged with silanized glass-wool plugs.

Preparation of cation-exchange resin

(a) Place the resin (*ca.* 450 g) (Amberlite IR-120-8X) in a 2000-ml erlenmeyer flask. The resin can be either new resin or resin used in previous determinations. Cover

the resin with sufficient deionized water to form a slurry and stir for *ca.* 1 h. Allow the resin to settle and decant off the water.

(b) Cover the resin with 7 *N* NH_4OH, stir for 1 h, allow the resin to settle and decant off the NH_4OH. Repeat this step two more times, then wash the resin with deionized water in the same manner until approximately neutral.

(c) Regenerate the resin to the H^+ form by adding sufficient 3 *N* HCl to form a slurry and stir for *ca.* 1 h. Allow the resin to settle and decant off the 3 *N* HCl. Repeat this step two more times, then wash the resin with deionized water in the same manner until approximately neutral. Keep the resin wet with sufficient deionized water to maintain a thick slurry.

Hydrolysis procedure

(a) Samples containing 20–25 mg of protein were weighed into 25 × 200 mm Pyrex glass screw-top culture tubes.

(b) 25 ml of 6 *N* HCl or constant-boiling HCl were added to the sample.

(c) A glass "T" arrangement with a rubber stopper fitted on the vertical portion of the "T" was fastened into the tube. To one arm of the "T" was fastened a piece of amber gum rubber vacuum tubing leading to a vacuum pump and to the other arm was fastened a piece of amber gum rubber surgical tubing leading to a nitrogen gas supply. A Whitey valve was placed in the vacuum line so that the vacuum and nitrogen pressure could be regulated on the tube.

(d) The pressure inside the hydrolysis tube was reduced to *ca.* 0.2 mmHg via the vacuum pump and the culture tube containing the hydrolysis mixture was placed in an ultrasonic bath. To remove dissolved air, the sample was repeatedly flushed with nitrogen gas. As a general rule this was done three to five times.

(e) After the dissolved air was removed, the Whitey valve was partially closed so that only a small amount of nitrogen gas would flow through. The nitrogen was slowly turned on such that a slight nitrogen pressure was formed in the tube. The glass "T" was then quickly removed and a PTFE-lined screw cap placed on the tube and tightened.

(f) The hydrolysis tube was placed in either a 110 ± 2° oven for 21 h or 145° for 4 h, then removed from the oven, and allowed to cool to room temperature.

(g) The sample hydrolysate solution was mixed and filtered through glass-fiber paper into a 50-ml volumetric flask, brought to volume and mixed.

Cation-exchange cleanup of hydrolysates

(a) An appropriate size aliquot containing approximately 10 mg of protein was placed into a 125-ml S round-bottom flask. 0.5 mg (5 ml of 0.10 mg/ml in 0.1 *N* HCl) of internal standard (tranexamic acid) was added and the sample solution then evaporated to dryness with a rotary evaporator under vacuum in a 60° water-bath.

(b) The amino acids were dissolved in 10 ml of 0.1 *N* HCl and placed onto a 7-ml cation resin bed of Amberlite CG-120 (H^+), 100–200 mesh, in a 15 × 150 mm ion-exchange column.

(c) The solution was passed through the resin bed at a rate of 1 ml/min followed by two 10-ml volumes of deionized water at a rate of 3 ml/min and three 10-ml volumes of deionized water at full flow. At no time was the liquid level allowed to fall below the top of the resin bed.

(d) The amino acids were eluted from the column with two 10-ml volumes of 7 *N* NH_4OH eluent at a flow-rate of 3 ml/min followed by 5 ml of deionized water at the same flow-rate. The eluate was collected in a 125-ml S flat-bottom flask.

Sample derivatization, esterification and acylation

(a) The alkaline eluate was evaporated to dryness under vacuum with a rotary evaporator in a 60° water-bath.

(b) Approximately 15 ml of *n*-butanol–3 *N* HCl (1.5 ml/mg protein) were added, a magnetic stirring bar was placed in the esterification flask and the flask was closed with a glass drying tube which contained drierite. Two lead rings were placed over the neck of the flask to prevent the flask from falling over.

(c) The flask was placed in an ultrasonic bath for 1 min and then into a 100 ± 2° oil-bath for 15 ± 1 min. The solution was mixed on a magnetic stirring hot plate during the 15-min esterification period.

(d) Following esterification, the flask was allowed to cool, then the *n*-butanol–3 *N* HCl was removed under vaccum in a 60 ± 5° water-bath.

(e) Approximately 10 ml of dry methylene chloride were added to azeotrope any remaining water and then removed under vacuum in a 60° water-bath. The flask was then cooled.

(f) Approximately 2 ml of dry methylene chloride and 1 ml of TFAA were added to the flask and mixed. The sample was then transferred into two 8-ml culture tubes and capped with PTFE-lined screw-caps (one sample as reserve).

(g) The sample was acylated at 150 ± 2° for 5 min and not exceeding 6 min. Following acylation the derivatized amino acids were placed in a freezer at 0° until chromatography was performed.

Sample preparation of blood plasma (free amino acids)

(a) Approximately 20 ml of saturated picric acid solution were placed in a 50-ml polypropylene centrifuge tube. Exactly 5.00 ml of plasma and 1.00 ml of 0.10 mg/ml tranexamic acid in 0.1 *N* HCl solution (internal standard) were added and the contents mixed.

(b) The solution was centrifuged for 15 min at 2400 *g*. Following centrifugation, the supernatant was decanted onto a 4-ml resin bed of Amberlite CG-120 (H^+) 100–200 mesh and allowed to pass through the resin bed at 1 ml/min. Subsequent cleanup and derivatization steps were the same as those used in the protein hydrolysate cleanup procedure.

For smaller plasma aliquots (0.4 and 0.1 ml) the same procedure was used only lesser volumes, smaller centrifuge tubes, and smaller columns were used. The ratio of 1 part sample to 4 parts picric acid was used. 12-ml centrifuge tubes were used and disposable Pasteur pipets containing glass-wool plugs and approximately 1 to 2 ml of cation-exchange resin were used as columns. Samples were washed with lesser amounts of water (10–20 ml) and eluted with 1 to 2 ml of 7 *N* NH_4OH into micro reaction vials. The eluates were evaporated under an infrared (IR) lamp, esterified with 250 to 500 μl of *n*-butanol–3 *N* HCl for 30 min at 100°, and acylated with 250–500 μl of TFAA–dichloromethane solution (1:9) for 5 min at 150°.

The Sol-Vent system

A major difficulty associated with the analysis of low levels of amino acids is the sensitivity required on the gas chromatograph. To circumvent this problem one must either concentrate the amino acid derivatives thus risking the possibility of breakdown of derivatives, insolubilization, volatilization loss; or inject larger volumes onto the gas chromatograph. The injection of large volumes of sample solution onto the EGA column causes a great deal of tailing of the trifluoroacetic acid (TFA) peak thus masking the first amino acids eluted off the column and causing integration difficulties. Also, the life of the column packing is drastically shortened due to interaction of the substrate with TFA and TFAA. The Sol-Vent is an injection port venting device which eliminates this problem. A small pre-column of a mixed-OV-siloxane packing is placed in front of the normal analytical EGA column. As the sample is injected a timer is activated which opens a solenoid valve for a prescribed time period allowing the solvent and other volatiles to vent to the atmosphere. At the end of the time period the valve closes, the temperature program is started, and the analysis performed in a normal manner. Retention of the amino acids is based on selection of the pre-column packing and its length, initial temperature, venting time, and carrier gas flow-rate. The Sol-Vent system allows one to make large injections (100 μl) of sample solution resulting in increased sensitivity and better baseline stability.

A diagrammatic representation of an improved Sol-Vent system is presented in Fig. 1.

This venting system incorporates the following features.

(a) An electronically timed venting period ranging from 6 to 150 sec. During this time solvent is allowed to escape from the gas chromatographic column.

(b) A "cold spot" is incorporated into the analytical column to retain compounds which are less volatile than the solvent in which they were dissolved. The "cold spot" is accomplished by an air stream directed on a 10-mm section of the GLC column. The temperature differential between the "cold spot" and the column oven is 25 to 75°. This temperature differential is designed for column temperature parameters ranging from 50 to 300°. Thumbwheel switches are used to select the desired "cold spot" temperature. After the cooling process is initiated, a pilot light indicates when the sample is to be injected.

(c) A carrier gas system which maintains a constant flow of gas through the detector during the venting period. This is accomplished via a pressure regulator on the Sol-Vent carrier gas input with a metering valve on the exit of the instrument.

The Sol-Vent can be used to vent the solvent from a GLC analytical column under isothermal and temperature programmed conditions. Another important feature is that the Sol-Vent can be interfaced with gas chromatographic detectors which are sensitive to flow disequilibrium, for example the thermionic and electron-capture detection systems.

RESULTS AND DISCUSSION

The primary purpose of this study was to develop procedures for sample preparation applicable to biologicals for amino acid analysis which were accurate, precise, and rapid. As Gehrke and others have published considerable data demonstrating the accuracy and precision of the GLC method *versus* classical cation-ex-

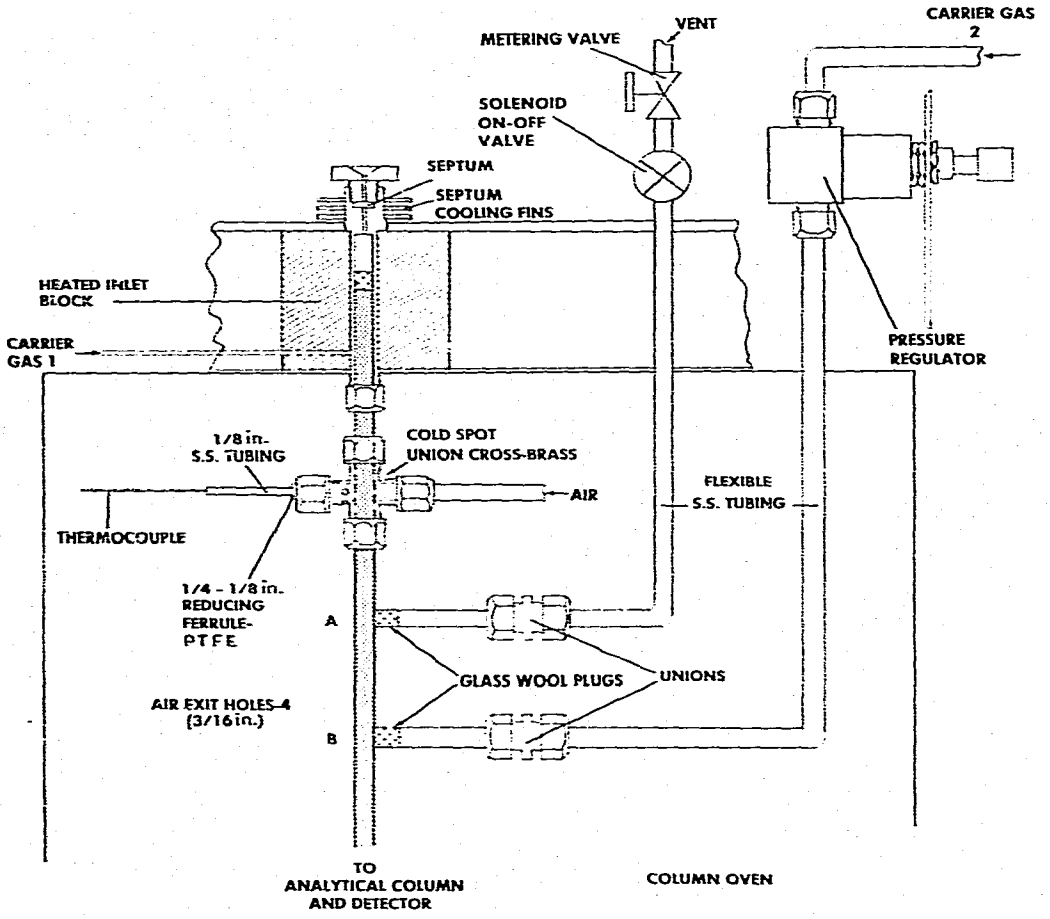

Fig. 1. GLC Sol-Vent system.

change chromatography, less effort was made to compare the two procedures in terms of quantitation. Tables I–III give the data obtained on three types of samples: a pure protein, a plant material, and a material of animal origin. The types of hydrolysis used were 21 h at 110°, and for 4 h at 145° with 6 *N* HCl. The samples were sonicated under vacuum prior to the 21-h and 4-h hydrolysis methods to aid in the removal of dissolved air from the sample solution prior to it being placed in the hydrolysis oven. These methods were compared to a 4-h hydrolysis at 145° in which sonication was not used. The data show that the 21-h and 4-h hydrolysates with sonication agree very closely in total amino acid content with the exception of the fish meal. The 4-h hydrolysis of fish meal gave a total amino acid content approximately 8% higher than the 21-h hydrolysis. Values for individual amino acids were also consistently higher. The 4-h hydrolysis with sonication resulted in higher values for isoleucine and equally as good a value for methionine and cystine. Slightly lower values were obtained for threonine and serine. Data comparing the 4-h hydrolysis with and without the use of sonication prior to heating show that methionine and cystine were somewhat

TABLE I

GLC AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS*
Values are given in % (w/w).

Amino acid	*21-h hydrolysis with sonication***			*4-h hydrolysis with sonication***			*4-h hydrolysis without sonication***		
			Av.			Av.			Av.
Alanine	6.74	7.02	6.88	6.80	6.89	6.85	6.80	6.75	6.78
Valine	6.52	6.37	6.45	6.59	6.55	6.57	6.47	6.28	6.38
Glycine	1.48	1.52	1.50	1.50	1.48	1.49	1.47	1.45	1.46
Isoleucine	1.48	1.43	1.46	1.98	2.13	2.06	2.06	2.06	2.06
Leucine	1.75	1.77	1.76	1.66	1.64	1.65	1.63	1.64	1.64
Proline	2.91	3.02	2.97	3.00	2.91	2.96	3.01	3.02	3.01
Threonine	6.79	6.90	6.85	6.86	6.69	6.78	6.57	6.81	6.69
Serine	8.08	8.53	8.31	7.72	8.19	7.96	7.71	7.51	7.61
Methionine	2.77	2.86	2.82	2.70	2.79	2.75	2.60	2.65	2.63
Phenylalanine	3.16	3.17	3.17	3.14	3.03	3.09	3.00	3.18	3.09
Aspartic acid	12.37	12.59	12.48	12.49	12.52	12.51	12.48	12.36	12.42
Glutamic acid	12.19	12.21	12.20	12.06	12.25	12.16	12.36	12.21	12.29
Tyrosine	6.73	6.97	6.85	6.77	6.74	6.76	6.40	6.74	6.57
Lysine	9.64	9.86	9.75	9.71	9.50	9.61	9.69	9.40	9.55
Histidine	3.52	3.57	3.55	3.53	3.36	3.45	3.45	3.45	3.45
Arginine	4.41	4.46	4.44	4.50	4.34	4.42	4.56	4.69	4.63
Cystine	4.96	4.98	4.99	4.95	4.89	4.92	4.68	4.74	4.71
Total			96.42			95.99			94.97
Average σ			0.09			0.09			0.09
Average relative standard deviation			1.66%			1.84%			1.45%

* Ribonuclease (bovine pancreas), Lot No. V1050 obtained from Mann Research Labs.
** Two independent hydrolysates.

lower for the ribonuclease and fish meal when sonication was not used. The cystine data on corn was also quite variable indicating degradation occurred during hydrolysis due to reaction with dissolved air.

Table IV shows the data obtained on the analysis of various size aliquots of human blood plasma. The data indicate the procedure works quite well with various levels of amino acids ranging from 1.4 mg to 26 μg of total free amino acids.

Table V shows the data obtained using the Sol-Vent device in conjunction with the EGA column. Good agreement was obtained on practically all the amino acids as compared to the data obtained without the use of the Sol-Vent system (Tables I–IV).

Table VI gives the data on the recovery of added methionine and cystine to various types of samples containing different amounts of protein and carbohydrate. Methionine and cystine were added to the 6 *N* HCl sample solution prior to sample preparation for the hydrolysis. Hydrolysis was made for 4 h at 145° with 6 *N* HCl. Sonication was used to aid in removing dissolved air. Good recovery was obtained on all samples except the yam which contained more than 95% of carbohydrates and starch. In another experiment, 2 mg of each amino acid was added to 5 ml of urine then taken through the classical ion-exchange cleanup and GLC method and the re-

TABLE II

GLC AMINO ACID ANALYSIS OF FISH MEAL AS A FUNCTION OF HYDROLYSIS

Values are given in % (w/w).

Amino acid	*21-h hydrolysis with sonication*[a]			*4-h hydrolysis with sonication*[a]			*4-h hydrolysis without sonication*[a]		
			Av.			Av.			Av.
Alanine	3.93	3.95	3.94	4.13	4.30	4.22	4.16	4.22	4.19
Valine	2.66	2.72	2.69	3.27	3.40	3.34	3.22	3.25	3.24
Glycine	3.59	3.65	3.62	3.92	3.98	3.95	4.00	3.99	4.00
Isoleucine	2.41	2.32	2.37	2.96	3.00	2.98	2.83	2.84	2.84
Leucine	4.38	4.33	4.36	4.72	4.89	4.81	4.73	4.68	4.71
Proline	2.43	2.36	2.40	2.46	2.52	2.49	2.53	2.51	2.52
Threonine	2.32	2.30	2.31	2.24	2.36	2.30	2.21	2.19	2.20
Serine	1.99	2.01	2.00	1.88	1.78	1.83	1.75	1.76	1.76
Methionine	1.36	1.34	1.35	1.61	1.69	1.65	1.40	1.47	1.44
Hydroxy-proline	0.45	0.49	0.47	0.53	0.49	0.51	0.48	0.48	0.48
Phenylalanine	2.29	2.42	2.36	2.66	2.75	2.71	2.59	2.67	2.63
Aspartic acid	5.17	5.33	5.25	5.66	5.76	5.71	5.78	5.83	5.81
Glutamic acid	8.73	8.65	8.69	8.90	9.28	9.09	8.64	9.02	8.83
Tyrosine	1.89	1.86	1.88	2.08	2.13	2.11	2.04	2.02	2.03
Lysine	4.35	4.17	4.26	4.49	4.58	4.54	4.34	4.38	4.36
Histidine	1.72	1.88	1.80	1.65	1.78	1.72	1.76	1.89	1.83
Arginine	3.00	3.25	3.13	3.12	3.20	3.16	2.99	3.27	3.13
Cystine	0.11	0.13	0.12	0.26	0.24	0.25	0.12	0.11	0.12
Total			53.10			57.36			56.12
Average σ			0.06			0.07			0.05
Average relative standard deviation			2.63%			2.74%			1.91%

[a] Two independent hydrolysates.

coveries determined. The recoveries ranged from 83 to 102% for 16 different amino acids.

Figs. 2–9 show the chromatograms obtained on the GLC analysis of ribonuclease, fish meal, corn, and blood plasma. The chromatography was performed on two columns, a 0.65% stabilized EGA on 80–100 mesh Chromosorb W AW, and a 2% OV-17 – 1% OV-210 on 100–120 mesh Gas-Chrom Q. The two column separation has the advantage of allowing confirmation of certain amino acids and in some cases the determination of non-protein amino acids. Complete baseline separation was obtained for all of the amino acids and no prevalent extraneous peaks appeared. Figs. 10–13 demonstrate the use of the Sol-Vent with the EGA column. When the Sol-Vent instrumentation is used, the methylene chloride and TFA peak are completely eliminated giving a smooth baseline resulting in greater accuracy of integration. This is particularly apparent on the chromatogram of human blood plasma. When the Sol-Vent device was used, the solvent and TFA peak were completely eliminated resulting in better integration values and better results.

Fig. 8 presents a GLC analysis which corresponds to injection of only 5 μl of

TABLE III

GLC AMINO ACID ANALYSIS OF CORN AS A FUNCTION OF HYDROLYSIS

Values are given in % (w/w).

Amino acid	*21-h hydrolysis with sonication*[a]			*4-h hydrolysis with sonication*[a]			*4-h hydrolysis without sonication*[a]		
			Av.			*Av.*			*Av.*
Alanine	0.64	0.65	0.65	0.67	0.69	0.68	0.65	0.66	0.66
Valine	0.50	0.44	0.47	0.49	0.50	0.50	0.47	0.50	0.48
Glycine	0.41	0.42	0.41	0.43	0.43	0.43	0.40	0.42	0.41
Isoleucine	0.33	0.29	0.31	0.32	0.33	0.33	0.32	0.31	0.32
Leucine	0.92	0.87	0.90	0.89	0.91	0.90	0.87	0.89	0.88
Proline	0.76	0.78	0.77	0.81	0.82	0.82	0.78	0.81	0.80
Threonine	0.35	0.34	0.35	0.33	0.33	0.33	0.34	0.33	0.34
Serine	0.50	0.48	0.49	0.45	0.40	0.43	0.47	0.43	0.45
Methionine	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Hydroxy-proline	0.03	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.03
Phenylalanine	0.40	0.41	0.41	0.43	0.42	0.43	0.41	0.42	0.42
Aspartic acid	0.83	0.84	0.84	0.85	0.85	0.85	0.84	0.84	0.84
Glutamic acid	1.69	1.68	1.69	1.76	1.76	1.76	1.79	1.76	1.78
Tyrosine	0.32	0.32	0.32	0.31	0.30	0.31	0.30	0.30	0.30
Lysine	0.39	0.39	0.39	0.40	0.40	0.40	0.39	0.40	0.40
Histidine	0.28	0.26	0.27	0.28	0.25	0.27	0.22	0.23	0.23
Arginine	0.50	0.49	0.50	0.55	0.57	0.56	0.54	0.55	0.55
Cystine	0.10	0.12	0.11	0.09	0.11	0.10	0.11	0.06	0.09
Total			9.08			9.30			9.15
Average σ			0.01			0.01			0.01
Average relative standard deviation			3.05%			2.38%			4.51%

[a] Two independent hydrolysates.

plasma onto the column. At the relatively high sensitivity required and without use of the Sol-Vent device for this analysis, the TFA peak is large and causes difficulty in obtaining accurate peak integration. With use of the Sol-Vent device, as seen in Fig. 13, the solvent and TFA peak are eliminated, allowing increased accuracy of peak integration. Thus the Sol-Vent system is particularly advantageous for the analysis of samples at the nanogram level, and has been extensively used by these authors in the analyses for water extractable organic material in the returned lunar samples[10,12–18]. All experimental details of instrumentation and chromatography are presented in our Lunar Analysis papers.

Comments on the method

(1) A water aspirator may be substituted for the vacuum pump when pulling a vacuum on the sample solution during sonication. A good vacuum, however, is important and should not vary from day to day.

(2) The protein sample should be completely wetted in the 6 *N* HCl prior to application of the vacuum. This can be facilitated with sonication. Otherwise, if the

TABLE IV

GLC AMINO ACID ANALYSIS OF HUMAN BLOOD PLASMA*

Amino acid	*mg/100 ml of plasma*						
	*5-ml aliquot***			*0.4-ml aliquot***			*0.1-ml aliquot*
			Av.			Av.	
Alanine	2.6	2.6	2.6	2.5	2.5	2.5	2.4
Valine	2.2	2.3	2.3	2.3	2.3	2.3	2.5
Glycine	1.9	1.9	1.9	1.8	1.8	1.8	1.8
Isoleucine	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Leucine	1.5	1.5	1.5	1.4	1.4	1.4	1.4
Proline	3.3	3.3	3.3	3.2	3.3	3.3	3.2
Threonine	1.8	1.8	1.8	1.6	1.6	1.6	1.4
Serine	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Methionine	0.2	0.2	0.2	0.1	0.1	0.1	trace
Hydroxyproline	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phenylalanine	0.8	0.8	0.8	0.9	1.1	1.0	1.0
Aspartic acid	0.6	0.6	0.6	0.6	0.6	0.6	0.8
Glutamic acid	4.5	4.5	4.5	4.5	4.5	4.5	5.0
Tyrosine	0.9	0.9	0.9	1.0	0.8	0.9	1.2
Lysine	2.5	2.5	2.5	2.5	2.4	2.5	2.4
Histidine	0.7	0.6	0.7	0.6	0.6	0.6	0.3
Arginine	1.5	1.3	1.4	1.6	1.1	1.4	1.9
Total			27.2			26.7	27.5

* Protein precipitated with saturated picric acid followed by cation-exchange cleanup.
** Two independent analyses performed on different days.

TABLE V

COMPARISON OF AMINO ACID ANALYSIS BY GLC WITH THE SOL-VENT SYSTEM

All of the data under vented (V) were obtained by one laboratory and the results for non-vented (NV) were obtained independently by another laboratory at another time.

Amino acid	*% (w/w)*						*mg/100 ml*	
	Ribonuclease		*Fish meal*		*Corn*		*Blood plasma**	
	V***,***	NV§	V***,***	NV§	V***,***	NV§	V***	NV§
Alanine	6.54	6.85	4.11	4.22	0.64	0.68	2.4	2.4
Valine	6.86	6.57	3.30	3.34	0.48	0.50	2.7	2.5
Glycine	1.72	1.49	3.79	3.95	0.41	0.43	2.3	1.8
Isoleucine	2.11	2.06	2.95	2.98	0.33	0.33	0.8	0.7
Leucine	1.87	1.65	4.70	4.81	0.86	0.90	1.4	1.4
Proline	2.98	2.96	2.51	2.49	0.79	0.82	3.4	3.2
Threonine	7.00	6.78	2.22	2.30	0.32	0.33	1.5	1.4
Serine	8.35	7.96	1.80	1.83	0.46	0.43	1.4	1.4
Methionine	2.45	2.75	1.40	1.65	0.17	0.17	0.2	trace
Hydroxyproline	0.00	0.00	0.54	0.51	0.02	0.03	0.2	0.1
Phenylalanine	3.16	3.09	2.67	2.71	0.43	0.43	0.8	1.0
Aspartic acid	12.61	12.51	5.60	5.71	0.84	0.85	0.8	0.8
Glutamic acid	11.73	12.16	8.57	9.09	1.72	1.76	5.3	5.0
Tyrosine	6.23	6.76	2.00	2.11	0.31	0.31	1.0	1.2
Lysine	8.88		4.35	4.54	0.39	0.40	2.6	2.4

* Data obtained on 0.1 ml of blood plasma.
** Data obtained on a single 4-h hydrolysate at 145° on which ultrasonication was used while preparing the sample solution for hydrolysis.
*** Analysis with Sol-Vent System.
§ Analysis without venting.

protein floats on the surface of the HCl, it may be lost during the vacuum removal of dissolved air from the tube.

(3) The temperature of the hydrolysis oven should be maintained at 145 ± 2° and the sample hydrolyzed for at least 4 h ± 5 min.

(4) Filtering of sample hydrolysates should be done only through glass-fiber paper. Other filter-papers could result in losses through adsorption of amino acids on the paper.

TABLE VI

RECOVERY OF METHIONINE AND CYSTINE

Methionine and cystine were added prior to 4-h hydrolysis at 145° on which sonication and partial vacuum were used to remove air.

Sample	*Methionine*			*Cystine*		
	Added (mg)	*Recovered (mg)*	*Recovery (%)*	*Added (mg)*	*Recovered (mg)*	*Recovery (%)*
Fish meal						
65% protein	1.00	1.02	102	1.00	0.99	99
Broiler feed						
18% protein	2.00	2.04	102	2.00	2.03	102
Corn						
10% protein	2.00	1.95	98	2.00	2.05	103
Yams						
1.2% protein	2.00	2.15	108	2.00	1.32	66

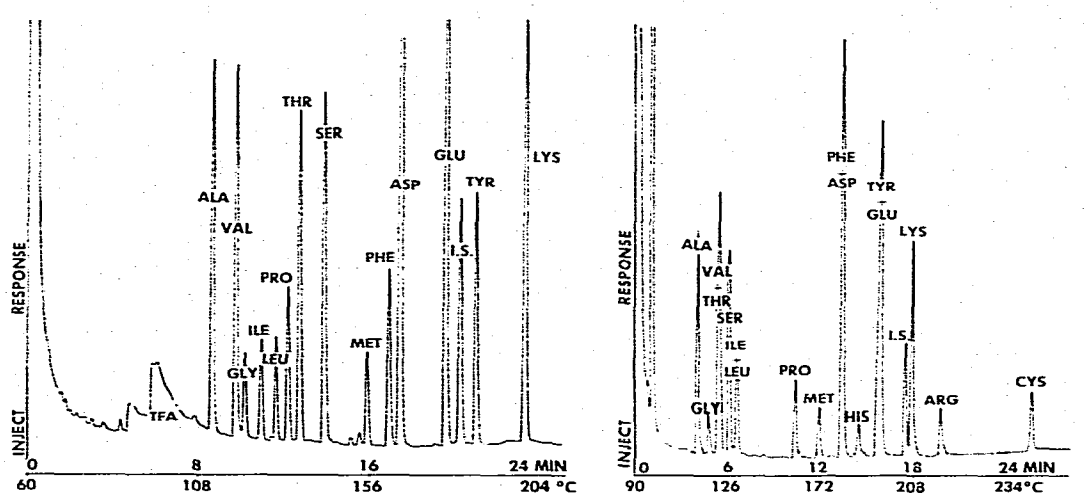

Fig. 2. GLC analysis of ribonuclease N-TFA *n*-butyl esters; without Sol-Vent. Column: 0.65% EGA on 80–100 mesh Chromosorb W AW, 1.5 m × 4 mm I.D. glass. Conditions: sample, 10.56 mg; final volume, 2 ml; injected, 3 µl (*ca.* 15 µg, total); attenuation, $2 \cdot 10^{-9}$ a.f.s.; initial temperature, 60°; program rate, 6°/min; final temperature, 210°; I.S., tranexamic acid.

Fig. 3. GLC analysis of ribonuclease N-TFA *n*-butyl esters; without Sol-Vent. Column: 2% OV-17, 1% OV-210 on 100–120 mesh Gas-Chrom Q, 1.5 m × 4 mm I.D. glass. Conditions: sample, 10.56 mg; final volume, 3 ml; injected, 5 µl (*ca.* 17 µg, total); attenuation, $16 \cdot 10^{-10}$ a.f.s.; initial temperature, 90°; program rate, 6°/min; final temperature, 235°; I.S., tranexamic acid.

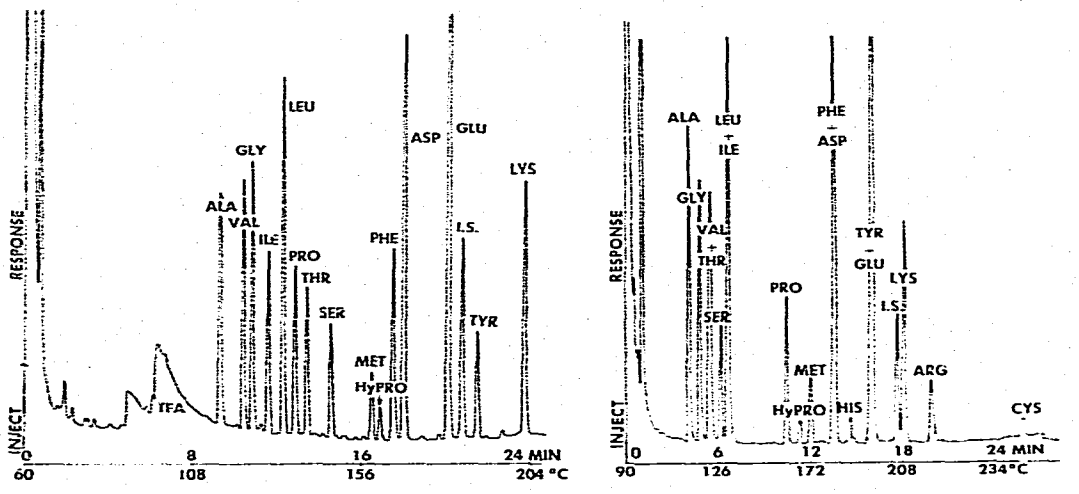

Fig. 4. GLC analysis of cation-exchange cleaned amino acids in fish meal hydrolysate; without Sol-Vent. Column, as in Fig. 2. Sample, 16.76 mg; injected, 2 μl (*ca.* 17 μg, total). Further conditions, as in Fig. 2.

Fig. 5. GLC analysis of cation-exchange cleaned amino acids in fish meal hydrolysate; without Sol-Vent. Column, as in Fig. 3. Sample, 16.76 mg; injected, 5 μl (*ca.* 15 μg, total). Further conditions, as in Fig. 3.

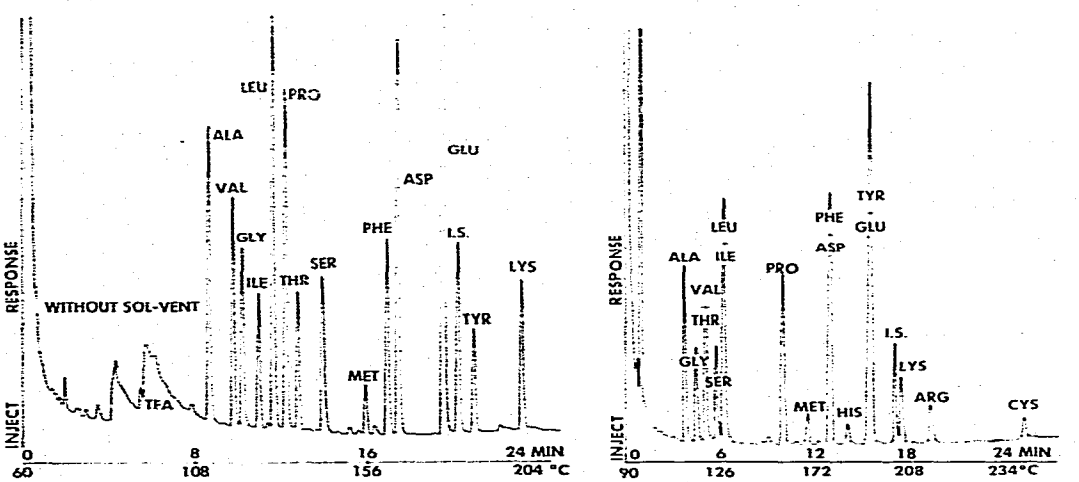

Fig. 6. GLC analysis of cation-exchange cleaned amino acids in corn hydrolysate; without Sol-Vent. Column, as in Fig. 2. Sample, 110.72 mg; injected, 3 μl (*ca.* 150 μg, total). Further conditions, as in Fig. 2.

Fig. 7. GLC analysis of cation-exchange cleaned amino acids in corn hydrolysate. Column, as in Fig. 3. Sample, 110.72 mg. Further conditions, as in Fig. 3.

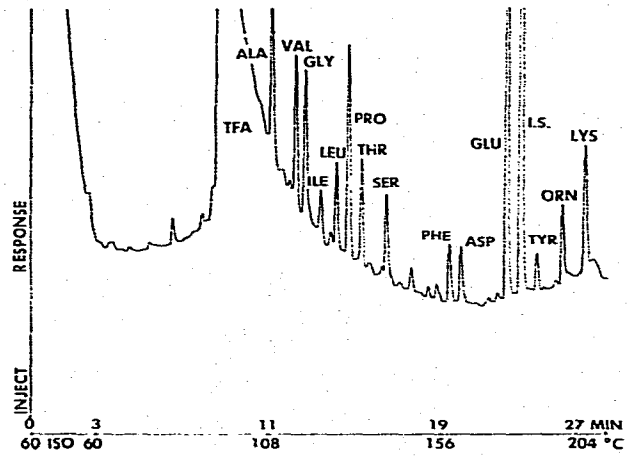

Fig. 8. GLC analysis of cation-exchange cleaned amino acids in human blood plasma deproteinized with picric acid: without Sol-Vent. Column, as in Fig. 2. Sample, 0.1 ml plasma; final volume, 0.1 ml; injected, 5 μl; attenuation, $1 \cdot 10^{-10}$ a.f.s.; initial hold, 3 min. Further conditions, as in Fig. 2.

(5) Protein hydrolysates should not be left in strong acid solutions for long periods of time. The sample aliquot should be drawn as soon as possible and the HCl removed.

(6) The 6 *N* HCl:sample ratio must be maintained at least 100:1, w/w, for complete hydrolysis.

(7) In cases as for the analysis of free amino acids in plant material or feces the addition of an extra wash with 80% ethanol or 50% acetone through the cation-exchange cleanup column prior to the last water wash is useful.

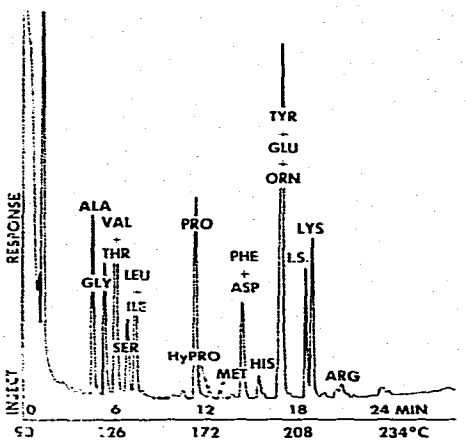

Fig. 9. GLC analysis of cation-exchange cleaned amino acids in human blood plasma deproteinized with picric acid: without Sol-Vent. Column, as in Fig. 3. Sample, 5 ml plasma; final volume, 0.7 ml; injected, 5 μl. Further conditions, as in Fig. 3.

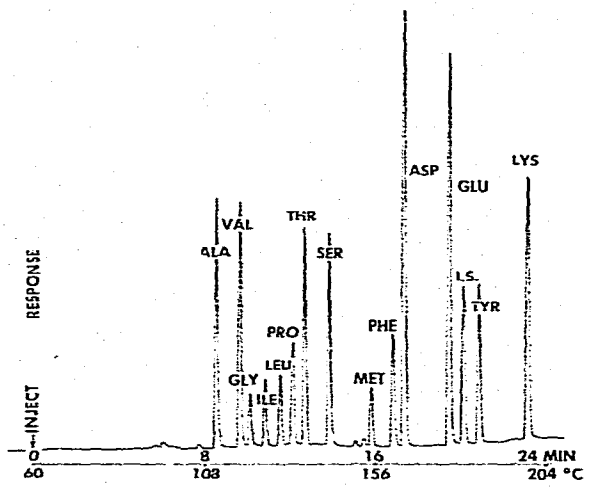

Fig. 10. GLC analysis of ribonuclease N-TFA *n*-butyl esters; with Sol-Vent. Column, as in Fig. 2. Injected, 2 μl (*ca.* 10 μg, total). Further conditions, as in Fig. 2.

(8) Precipitation of protein in plasma and serum with picric acid is recommended rather than trichloroacetic acid or sulfosalicylic acid because of less GLC interference. Membrane techniques or ultracentrifugation could be used. Cation-exchange columns should be washed with water until no visible picric acid remains.

(9) After the alkaline eluate has been taken to dryness, rigorous attempts to exclude moisture must be followed. Reagents must be dry, drying tubes should be left on the sample flasks, and acylation tubes capped as soon as possible.

(10) Methylene chloride can be kept anhydrous by storing in an all-glass in-

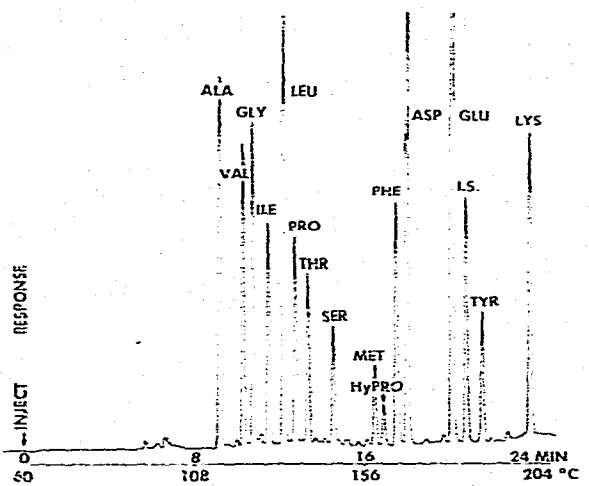

Fig. 11. GLC analysis of cation-exchange cleaned amino acids in fish mealhydrolysate; with Sol-Vent. Column, as in Fig. 2. Sample, 16.76 mg; injected, 2 μl (*ca.* 17 μg, total); vent time, 40 sec. Further conditions, as in Fig. 2.

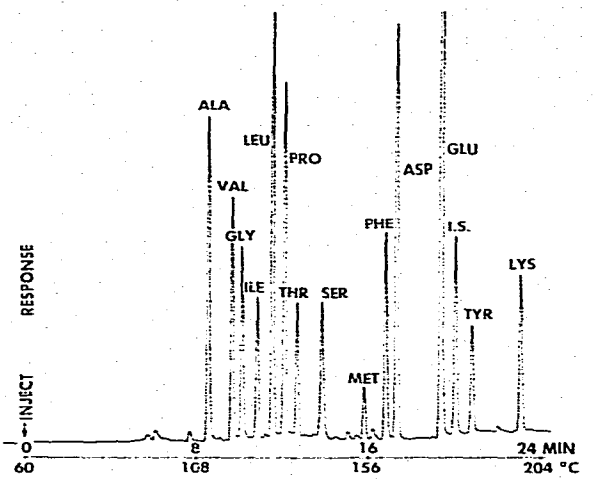

Fig. 12. GLC analysis of cation-exchange cleaned amino acids in corn hydrolysate; with Sol-Vent. Column, as in Fig. 2. Sample, 110.72 mg; injected, 3 μl (*ca.* 150 μg, total); vent time, 40 sec. Further conditions, as in Fig. 2.

verted top bottle over $CaSO_4$ or by storing over $CaSO_4$ in a pipettor bottle to which a drying tube containing calcium sulfate is attached to the air inlet.

(11) Metals must be excluded from the derivatization reagents and materials or methionine will be lost. Methionine forms insoluble salts with metals, particularly iron. Magnetic stirring bars are an excellent source of metal contamination. They should be soaked in 3 to 6 *N* HCl several hours prior to use.

(12) Temperatures of the esterfication and acylation baths should be maintained within $\pm$ 2° of 100° and 150°, respectively. Low temperatures result in decreased

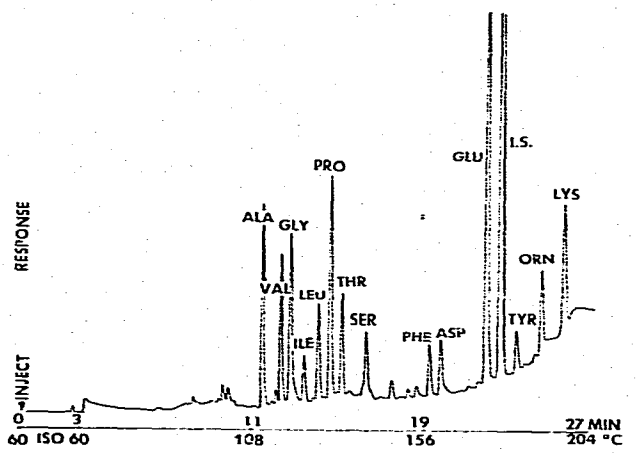

Fig. 13. GLC analysis of cation-exchange cleaned amino acids in human blood plasma deproteinized with picric acid; with Sol-Vent. Column, as in Fig. 2. Sample, 0.1 ml plasma; final volume, 0.1 ml; injected, 5 μl; attenuation, $1 \cdot 10^{-10}$ a.f.s.; vent time, 40 sec; initial hold, 3 min. Further conditions, as in Fig. 2.

values for isoleucine and leucine. Likewise, time periods for esterification and acylation should be monitored closely. Increased esterification time results in higher isoleucine values, and increased acylation time results in lower methionine, histidine, and cystine values. Temperature and time must be closely controlled.

Metal heating blocks and convection ovens should not be used as the heat-bath for derivatization and acylation due to non-uniformity in heat transfer. It is recommended that an oil-bath or glycerol-bath be used.

Gas chromatographs with metal transfer lines should also be avoided for amino acid analysis. Tyrosine, serine and threonine are easily decomposed on hot metal surfaces.

(13) Under *Sample derivatization, esterification and acylation* (step f) the esterified sample was divided into two parts, one maintained as a reserve.

(14) Cleaning of glassware with chromic acid should be avoided if possible. If chromic acid must be used, rinse the glassware well with hot 1 to 3 *N* HCl followed by exhaustive rinsing with distilled water.

(15) Purity of reagents should periodically be checked particularly if small amounts of amino acids are to be analyzed. Procedural blanks should be run on a regular basis.

In nanogram analysis for amino acids each chemical reagent must be checked before and after derivatization of a procedural blank[10,12–18].

(16) The *n*-butanol–3 *N* HCl should be prepared by slowly bubbling HCl gas into the butanol. This reagent should be stored in a brown bottle with a PTFE stopper or PTFE-lined cap and kept under refrigeration.

(17) When preparing derivatives in micro reaction vials, drying is best accomplished with an IR lamp. Care should be taken not to overheat the sample derivatives. Vials should be removed from under the IR lamp as soon as they are dry. An acylating mixture of 9 parts methylene chloride to 1 part TFAA works very well for the small amounts of amino acids.

For procedural details, please refer to our papers on analysis of amino acids in lunar samples[12–18]. Baking or drying too long or at elevated temperatures of the esterified samples results in volatization losses of the derivatives.

(18) Internal standards other than tranexamic acid may be used. *n*-Butyl stearate elutes between tyrosine and lysine on the EGA column and between arginine and cystine on the mixed OV column. The derivative of *α*-aminopimelic acid [HOOC-$(CH_2)_3$CH(NH_2)-COOH] elutes between tyrosine and lysine on the EGA column and between lysine and arginine on the mixed OV column. Diaminopimelic acid can also be used but its derivative elutes a little too late on the EGA column to be useful. Ornithine is satisfactory if analysis is not required for histidine, arginine, and cystine, since ornithine does not separate from glutamic acid and tyrosine on the mixed phase OV column.

(19) Filters containing some type of material which will remove moisture and hydrocarbons should be placed on all gas lines to the instrument. Compounds such as $CaSO_4$, silica gel, and molecular sieve serve well. Nitrogen gas of the highest purity should be used.

(20) Acetonitrile is preferred as the solvent for the preparation of the EGA packing rather than the acetone. The vapor pressure and boiling point of acetone is considerably lower than that of acetonitrile. This results in too rapid a removal of

acetone from the packing causing uneven and incomplete distribution of the EGA liquid phase on the solid support.

(21) Conditioning of columns is done by programming from room temperature to upper limit temperature at a rate of 1–2°/min overnight. Columns should be conditioned with a nitrogen flow of 20–40 ml/min. The recommended conditioning temperature for the EGA column is 220° and the mixed phase OV is 250°. Prolonged conditioning of the OV column (2–5 days) at 250° results in some bleed off of OV-210 and tailing of amino acid derivatives. Do not hold an EGA column at 230° for more than 15 min after chromatography. Reduce the temperature to 140° for prolonged holding periods.

(22) Column life should range from 2–6 months depending on types of samples analyzed and column abuse. Many times a column can be regenerated by removing the front 6 to 8 in., repacking with new column material and reconditioning. A new EGA column is needed when the valine–glycine, proline–threonine, and methionine–hydroxyproline–phenylalanine groups cannot be separated. A new mixed phase OV column is needed when relative molar responses for arginine and cystine decrease. Large amounts of silanized glass-wool should not be used to plug the ends of the column to retain the packing. Use the minimum amount necessary to keep the packing in the column, usually about $^1/_4$-in. plug of glass-wool tightly packed into the column is sufficient.

(23) If columns are removed from the instrument, the ends should be capped during storage to exclude atmospheric moisture. When columns are placed back in the instrument a 1-h conditioning period should be sufficient.

(24) Good separations are obtained with both packings if 1.5 m × 2 mm columns are used instead of 1.5 m × 4 mm columns. One must remember, however, that flow-rates will generally be lower and amounts injected onto the columns generally less.

(25) Gas chromatographs which have stainless-steel transfer lines leading from the exit end of the column to the detector should be avoided. Breakdown of derivatives can occur. If this type of instrument must be used, replace the transfer line with a glass-lined stainless-steel interface.

CONCLUSIONS

The analysis of biological samples, *i.e.* ribonuclease, fish meal, urine, and corn with the method gave results in good agreement on hydrolysates (21 h at 110° *vs.* 4 h at 145°) with sonication and vacuum applied to remove dissolved air. Average relative standard deviations of 1.84% for ribonuclease, 2.74% (fish meal), and 2.38% (corn) were obtained. Also, the method worked well for analyses at the 1.4-mg to 26-μg level of total amino acids in blood plasma (5 ml to 0.1 ml), and good recoveries were obtained for amino acids added to urine. Excellent recovery of added methionine and cystine were obtained from fish meal, broiler feed and corn.

Blood plasma is analyzed for free amino acids by precipitating the protein with saturated picric acid followed by cation-exchange cleanup. Quantitation was improved for small amounts of amino acids (micrograms) with the use of a Sol-Vent device. This device allows one to inject large amounts of sample solution (100 μl) onto the GLC column, vent the solvent, and still retain the amino acids. Greater sensitivity and good base line stability were achieved.

In the analysis of amino acids the sample preparation techniques are more determinant than the methods of analyses used, whether they be classical ion-exchange chromatography, GLC or others. It has been our purpose to present the practical laboratory methodology and details that we have found reliable.

The sample preparation methods presented; hydrolysis of protein at 145°–4 h, cation-exchange cleanup, derivatization, along with a gas chromatographic solvent venting system, are most important and provide the means for reliable analysis of amino acids by GLC.

We are hopeful that these methods developed and used in our respective laboratories will prove useful to others.

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All of the above scientists contributed significantly to the development of this method.

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