Journal of Chromatography, 95 (1974) 189–212

CHROM. 7482

DETERMINATION OF AMINO ACID PROFILES IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY

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(Received March 11th, 1974)

SUMMARY

Based on solvent-free gas chromatography of the *n*-propyl, N-acetyl derivatives, procedures are described for the routine determination of amino acid profiles in biological samples including protein hydrolysates, plant tissue extracts, urines and sera. Derivatization is carried out in a 25-min two-step procedure. Use of a basic acylation environment allows derivatization of arginine and histidine. Chromatography using capsule injection on to a polar column with temperature programming gives resolution of 21 common amino acids within 15 min. Norleucine used as the internal standard provides quantitative capability. Sensitivity of the method permits quantitation at the nanomole level. Recoveries of amino acids added to sera ranged over 90–99%; an exception was arginine which gave 78%. Typical reproducibility data indicate that a coefficient of variation of 2–5% is attainable.

INTRODUCTION

The use of gas chromatography (GC) for the determination of amino acids is attractive because it provides quantitative results at high sensitivity, at a speed unequalled by other procedures. Indeed several GC procedures are detailed in the literature (*e.g.*, refs. 1–6). Common to these is the requirement to derivatize the amino acids in order to make them volatile, but stable enough for the GC analysis. The relative ease with which amino acids may be chemically modified to give many different derivatives[7,8] often leads to difficulty in choosing a derivative for a particular purpose. Where a particular amino acid is to be analyzed it is possible to select a derivative which will provide remarkable simplicity and speed in the analysis, as, for example, the procedure of Halpern *et al.*[9] for serum phenylalanine. Other possible derivatives, for example the N-dimethylaminomethylene alkyl esters[10], have great potential for amino acid analysis but insufficient evaluation has been published on their utility for the analysis of biological samples.

For routine determination of amino acid profiles, especially where accuracy, rapidity, economy, and simplicity of manipulation are prime requirements, the *n*-propyl, N-acetyl derivatives[4,5,8,11] warrant serious consideration. The reagents required are readily obtainable at high purity, are inexpensive and are not unduly sensitive to moisture. The derivatives are stable and are prepared in a two-step pro-

cedure that may be carried out in a screw-capped test tube. The use of a capped tube simplifies the manipulation. A single addition is made of the propylation reagent. In conjunction with a standard heating block there will be a temperature gradient along the tube which induces a refluxing action in the reagent. Furthermore, the pressure developed in the tube assists the reaction. Problems associated with moisture originating in the sample or from the reaction are obviated by use of a small amount of 2,2-dimethoxypropane as a water scavenger. An additional advantage is the commercial availability of pure derivatives allowing ready monitoring of recoveries and optimization of GC conditions.

The propylation is effected using propanolic HCl. The acylation reagent used is a more basic reagent than previously used for this purpose[5], consisting of acetic anhydride, triethylamine and acetone. The reagent effects acylation rapidly (within 2 min) and completely for most amino acids. Arginine and histidine, which have strongly bound HCl on the molecule following the propylation step, are also acylated with the mixed reagent used in the procedures that follow, thereby obviating the need for alternate procedures to determine these amino acids[5]. During the esterification, asparagine and glutamine are converted to the corresponding acids. Cystine is analyzed as cysteine, a reducing agent being used to effect preliminary reduction to cysteine in the sample. Organic reducing agents such as thioglycol are suitable but for this report stannous chloride was used.

Although dried protein hydrolysates may be derivatized and analyzed directly, most other biological samples require pretreatment to separate the amino acids from the matrix which may include protein, carbohydrates, salts, urea, lipids and peptides. Of several methods[9,11–14] most emphasize deproteinization. For this report a method is used based on that by Harris *et al.*[15] utilizing a cation-exchange resin for removal of cations present in the sample followed by preferential elution of the amino acids using an aqueous solution of ammonium hydroxide. The cation-exchange resin which was selected has high cross-linkage characteristics enabling deproteinization of the sample to be achieved by molecular exclusion chromatography.

GC of amino acid samples on capillary columns will be undoubtedly of increasing importance because of the speed and resolution of these columns. An indication of the potential inherent in the use of this type of column is given in the study by Jönsson *et al.*[16]. However, packed columns were used for this report.

GC on a single packed column of the *n*-propyl, N-acetyl amino acids has been considerably improved by the availability of a stable, polar, silicone liquid phase. Previously, complete resolution of the *n*-propyl, N-acetyl derivatives of all the protein amino acids was difficult to achieve on a routine basis, because it was necessary to use liquid phase mixtures[4,5,11] which were unsatisfactory for long-term routine use because of temperature limitations. The column used for this report was 3 ft. × 1/8 in. O.D. stainless steel. The packing was 0.31% Carbowax 20M, 0.28% Silar 5CP and 0.06% Lexan on Chromosorb W AW, 120–140 mesh. The temperature limit of this combination is better than 250° and allows repeated use of the column to this temperature without visible loss of resolution.

Injection of samples using solvent-free capsule injection (Model MS-41 Capsule Sampler)[17] was employed because of its advantages over syringe injection. Amongst others, these advantages include elimination of the septum and solvent, and greatly improved quantitative reproducibility for the compounds analyzed.

Additionally, it is possible to transfer, by successive washings, the total sample to the capsule for injection, thereby providing improved sensitivity for small samples.

The procedures were studied to determine derivatization reproducibility of pure amino acids and recoveries of amino acids from biological materials. Results obtained by the procedures were applied to a wide range of biological materials including protein hydrolysates, plant extracts and physiological samples. Emphasis has been given to analysis of sera and urine for amino acid metabolic disorders. GC analyses were computerized, which provided considerable flexibility and precision in handling the data. The final reports were expressed automatically in micromoles or milligrams for amino acid concentrations, as referenced against a common standard.

MATERIAL AND APPARATUS

Reagents

The following reagents were used: Dowex 50-X8 (H^+) 100–120 mesh (Sigma, St. Louis, Mo., U.S.A.); stannous chloride, analyzed reagent grade (Matheson, Coleman & Bell (East Rutherford, N.J., U.S.A.); ammonium hydroxide, analyzed reagent grade (Matheson, Coleman & Bell); dry air or nitrogen; ethyl acetate, spectroquality (Matheson, Coleman & Bell); acetic acid, glacial, analyzed reagent grade (J.T. Baker, Phillipsburgh, N.J., U.S.A.); acetic anhydride, 99+% (Matheson, Coleman & Bell); 2,2-dimethoxypropane, 99+% (Aldrich, Milwaukee, Wisc., U.S.A.); acetone, spectroquality (Matheson, Coleman & Bell); triethylamine (Matheson, Coleman & Bell); *n*-propyl alcohol* (Matheson, Coleman & Bell); anhydrous HCl*, technical grade (Scientific Gas Products, Edison, N.J., U.S.A.); amino acids, A grade (Calbiochem, La Jolla, Calif., U.S.A.); norleucine, A grade (Calbiochem) (for use as an internal standard); *n*-propyl, N-acetyl amino acids (Grafl Ass., Santa Clara, Calif., U.S.A.); α-chymotrypsin protein (bovine pancreas, cryst.), A grade (Calbiochem.); ovalbumin, cryst., grade V (Sigma); histone (calf thymus), Type II (Sigma); lysozyme, 3× cryst. (egg white), A grade (Calbiochem); ribonuclease, 5× cryst. (bovine pancreas), A grade (Calbiochem.); collagen (calf skin, acid soluble) (Sigma).

pH adjusting solution. A 50% solution of acetic acid in deionized water and containing 5.0 mg $SnCl_2$/100 ml was used for adjustment of sample pH. (The stannous chloride is introduced with this solution to reduce cystine present in the sample to cysteine.)

Propylation reagent. The propylation reagent, propanolic HCl, was made by passing anhydrous HCl into 200 g cooled *n*-propyl alcohol until the reagent was 8 *M* in HCl on a w/w basis. Stored at 4° in a polypropylene bottle the reagent lasts several months.

Acylation reagent. A mixture of acetone, triethylamine and acetic anhydride (5:2:1) was prepared daily. This reagent may discolor on standing but this will not have any effect on the acylation.

Preparation of resin column

The column was made by pushing a small quantity of glass wool well down

* See *Propylation reagent*, below.

into the stem of a pasteur pipet. The glass wool was not too firmly compacted to avoid reducing the flow-rate. 50 mg of Dowex 50-X8 (H^+) 100–120 mesh resin was washed (using deionized water) on to the glass wool. It is important that high-quality moist resin be used for the column, and that it should be in the H^+ form. Commercially available resin proved adequate without special regeneration being required but the stock container was kept tightly sealed to prevent loss of exchange capacity due to adsorption of ammonia from the laboratory air. Expended resin was regenerated by passing 2 ml 1 *N* HCl through the resin column at about three drops per second then washing the resin with deionized water to pH 6–7. A schematic of the column showing approximate positioning of the resin is given in Fig. 1. 50 mg of resin will provide approximately 150 *μ*equiv. exchange capacity. This is adequate for treatment of most types of samples. For example, 100 *μ*l serum contains approximately 1 *μ*mole amino acids and 15 *μ*equiv. of inorganic cations[15]. 50 mg of resin with 150 *μ*equiv. capacity represent a tenfold excess over requirements.

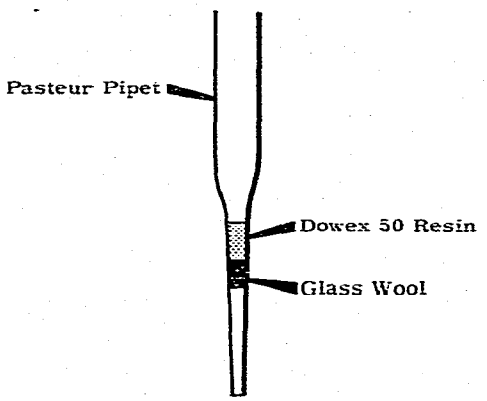

Fig. 1. Schematic of resin column illustrating positioning of the resin.

Standards. A stock solution of each amino acid including norleucine was made up in aqueous 0.1 *N* HCl at a level of 0.1 m*M*/ml. A working solution containing all the amino acids except norleucine was made up by combining equal aliquots of the individual amino acids, evaporating down the mixture to a small volume then diluting this with 0.01 *N* HCl to provide a level of 10 *μM*/ml for each amino acid.

Internal standard. Norleucine was used as the most convenient pure internal standard available. Working standards in 0.01 *N* HCl were prepared at a level of 10 *μM* norleucine/ml and at a level of 2 mg/ml. For results to be expressed in *μ*moles, the former is used, for results to be expressed in mg, the latter is used.

Standards were stored in capped polypropylene bottles and maintained at 4°.

Glassware

Glassware included 1-ml graduated pipets, 10-*μ*l disposable micropipets, 15 × 85 mm disposable glass culture tubes, 16 × 75 mm glass culture tubes with screw-cap (PTFE-lined) closures (Kimax No. 45066A) and 145-mm disposable pasteur pipets. For 10-*μ*l serum analyses, it was found useful to employ special reaction tubes, Reactivials, 3 ml, supplied by Pierce.

Equipment

The following equipment was used: heating block, Temp-blok (Scientific Products); manifold (for evaporation of derivatized samples); rotary evaporator, Buchi Rotavapor-R (Brinkman).

Gas chromatography

A Perkin-Elmer Model 3920 gas chromatograph with dual flame ionization detectors and linear temperature programming was used together with a Model 56 1-mV recorder. Injection was carried out with the Model MS-41 solvent-free capsule injection system. The PEP-2 Data Processor was used for automatic data reduction.

Column

The column used was 3 ft. × 1/8 in. O.D. stainless steel. This was packed with a mixed polar packing consisting of 0.31% Carbowax 20M (Perkin-Elmer), 0.28% Silar 5CP (Applied Science Labs., Rochester, N.Y., U.S.A.) and 0.06% Lexan (Perkin-Elmer) on Chromosorb W AW, 120–140 mesh. Prior to coating, the Chromosorb was heated to remove moisture at 400° for 1 h, then cooled. The packing was prepared by combining the required quantity of liquid phases in 140 ml of a chloroform–methanol mixture (85:15) and pouring the mixture on to 50 g of Chromosorb W AW contained in a 500-ml round-bottom flask. Then the solvent was cautiously evaporated off using a rotary evaporator adjusted to the lowest speed and a water-bath set at 90°. After filling the column it was conditioned with a helium flow of 12 ml/min at 250° for 2 h, then 16 h at 220°, or until the baseline was stable.

PROCEDURES

Sample requirements

Protein hydrolyzates. Acid hydrolyzates of pure protein were evaporated down with added internal standard (norleucine) to a dry residue and the derivatization procedure given below carried out. With a suitable quantity of protein (about 1–10 mg), it was possible to carry out the hydrolysis in a screw-cap tube used for all subsequent chemical manipulation, thereby obviating transfers.

Serum and urine samples. As with all complex samples, the amino acids require separation from some of the biological matrix. The method employed depends on adsorption of cations from a solution at pH 2–2.5 on to a Dowex 50 cation-exchange resin column. Non-ionic material and anions are washed through the column. Amino acids are preferentially eluted from the resin using 2 *N* NH_4OH. The procedure used was as follows.

Sample pretreatment

Serum or urine. (1) Transfer 0.1 ml of serum or 0.5 ml urine, 10 μl (=20 μg) of 2 mg/ml norleucine internal standard (see *Internal standard*) and 0.1 ml pH adjusting solution to a 16 × 75 mm tube. Mix gently. The pH should be 2–2.5 and is checked by spotting a minute amount of the mixture on to universal indicator paper. (2) Transfer mixture to resin column using a pasteur pipet for the transfer. Allow to pass through column at about one drop per 5 sec using very little air pres-

sure as, for example, from a rubber bulb. *Caution.* Do not allow column to run dry! (3) Wash sample tube with 0.5 ml deionized water and transfer the washing to the column. Pass through the resin and immediately follow this with 0.5 ml of deionized water. The flow-rate may be increased to about one drop per second. (4) The resin is eluted using 2 ml 2 *N* NH_4OH passed through the column at about one drop per second. The eluate is collected in a 16 × 75 mm screw-cap culture tube and evaporated to dryness using a rotary evaporator and a water-bath set at 90°. (A 4-cm length of 1-cm Tygon tubing is suitable for connecting the tube to the rotary evaporator.)

Plant samples. Leaf, root tissues and pulses were first extracted and cellulose and other fibrous or particulate material was removed. The sample was extracted with 70% ethanol. In this case, the plant samples were extracted by pulverizing a weighed quantity (1–10 g) of tissue with several volumes of aqueous 70% ethanol. The combined filtered extracts were flash-evaporated to a small volume and the volume made up with 70% ethanol so that a 1-ml extract was equal to 1 g of the original tissue. 100 µl of the extract were taken to dryness and 10 µl (20 µg) of internal standard were added. The residue was taken up in 0.5 ml deionized water and 0.5 ml pH adjusting solution. The pH was checked (pH 2.0–2.5) and processed as for serum.

Where a buffer (*e.g.*, phosphate buffer) containing cations is employed for the extraction, due consideration must be given to the exchange capacity of the resin. Generally, the resin quantity will be sufficient to take up cations in the sample. It is advisable to increase the resin quantity where other cations (*e.g.*, in a buffer) are added. The appropriate milliequivalent exchange capacity of the batch of resin used will provide the information for calculating this quantity.

Plant juices. Following centrifugation or filtration to remove debris, plant juices may be processed through the column directly after addition of internal standard and adjustment to pH 2–2.5. Because of species and varietal variation, citrus juices should be adjusted to the correct pH using 10% acetic acid which is added dropwise until the correct pH range is attained. A suitable volume of juice to use is 0.1 ml. The sample is processed through the resin and the eluate taken to dryness.

Derivatization

(1) To the dry eluate residue contained in a screw-cap 16 × 75 mm culture tube, add 50 µl (one drop) dimethoxypropane followed by 1 ml of propylating reagent. The dimethoxypropane scavenges moisture that may be present in the residue and removes water formed during esterification.

(2) Cap the tube firmly and heat it in a heating block at 110° for 20 min. Although several hundred reactions of this type have been processed without tube breakage, it is advisable to use a safety screen at this stage.

(3) Cool the tube briefly and open. Evaporate off excess reagent at 110° using a current of dry air or nitrogen. It is useful to hold moistened universal indicator paper just above the mouth of the tube to determine that all acid vapors have been evaporated off. Residual acid can react with the acylating reagent to produce an insoluble salt. This interferes mechanically with subsequent transfers and reduces the amount of reagent available for the reaction.

(4) Cool the tube briefly. Add 1 ml of acylating reagent. Cap the tube firmly.

Warm the tube for 30 sec at 60°. Cool the tube briefly. Evaporate the excess reagents at 60° using a current of dry air or nitrogen. (*Caution.* Derivatized material at this point is very volatile. Do not exceed the recommended temperature and use a gas flow not greater than 50 ml/min! Higher temperatures and gas flows will cause losses of several amino acids, particularly alanine and valine.)

(5) On completion of evaporation, no strong odor of acetic anhydride should be apparent. Cool the tube, take up the residue in 0.1 ml of anhydrous ethyl acetate. Cap the tube. The derivatized material is stable for several days at room temperature and several months when stored at 4°. 2- to 10-μl aliquots of the derivatized material are taken for the GC analysis.

GC analysis

The following instrument settings were used. The helium carrier gas flow-rate was 12 ml/min at an inlet pressure of 70 p.s.i. The injector temperature was 250° and the detector temperature was 300°. The oven was temperature programmed from 125° to 180° at 8°/min and 180° to 250° at 32°/min. Attenuation was set at 64×10. Injections were made by Perkin-Elmer MS-41 capsule system[17], evaporating off the solvent from the capsule before sealing and injecting. The capsule system was chosen because of its several advantages, including improved resolution, improved reproducibility and avoidance of solubility problems associated with the use of a solvent.

The GC performance was tested for resolution and sensitivity by injecting 1 μl of a mixture of pure derivatized amino acids containing 4 nmoles/μl of each derivative. A typical chromatogram is given in Fig. 2. Derivatized tryptophan was chromatographed isothermally at 250° on columns made from three different batches of packing in order to provide HETP data. Plate numbers for the 3-ft. columns ranged from 2,250 to 2,400 plates for the tryptophan derivative. The resolution of the amino acid standard was visually identical on the columns except that retention times on one column were somewhat greater for the later peaks.

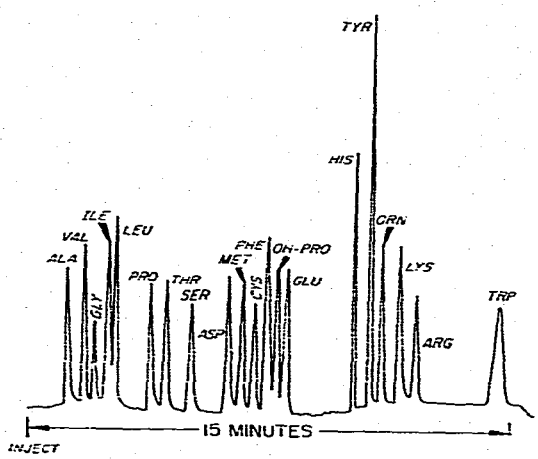

Fig. 2. Chromatogram obtained by injecting a mixture containing 4 nmoles of each derivatized amino acid.

The GC analyses were carried out using the GC parameters described above. Three columns were used for the analyses, it being convenient to use one for sera and urine analyses on a continuous basis, the others for plant extracts and protein hydrolysates. There was minimal variation in performance among columns. Throughout all the analyses reported here norleucine was used as an internal standard to provide quantitation capability and identification of other amino acids using retention times relative to the norleucine derivative. The analytical method for the computer used for the data reduction was obtained using standard amino acid mixtures taken through the complete procedures and gas chromatographed. The computer calculated the relative retention times (RRT) and response factors for each amino acid and applied this information for data reduction of all subsequent analyses.

It is of paramount importance to ensure that reagents used do not contribute unwanted volatiles to the GC analysis. Hence reagent blank runs were made to determine the suitability of the reagents used for this type of analysis. The purest obtainable amino acids were used for standards. It was found that several have other amino acids as impurities. Typical of this situation is the Grade A arginine used for the work reported here. When derivatized and chromatographed, it gave a small secondary peak corresponding in retention time to derivatized ornithine, as shown in Fig. 3. To determine whether some decomposition of arginine had occurred during derivatization, the free arginine was chromatographed by two-dimensional thin-layer chromatography (TLC) on silica. The chromatograms showed a small ninhydrin-positive, Sakaguchi-negative spot with R_f characteristics similar to ornithine. It was

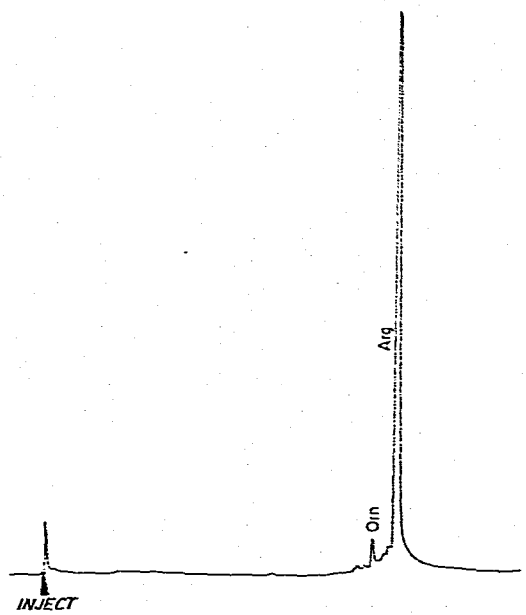

Fig. 3. Chromatogram of derivatized arginine. Injected sample size was 2 μg. Ornithine was present as a contaminant in the arginine. The remainder of the background indicates the low level of interference contributed by the reagents.

concluded that the secondary peak on the chromatogram was ornithine originating as an arginine impurity. The remainder of the chromatogram illustrates the type of background obtained with the reagents and procedure used for this report. Although several small peaks are discernible, they provide no serious interference problems.

RESULTS

Derivatization

To ascertain reproducibility of derivatization, fifteen aliquots of a mixed amino acid standard solution were transferred to the reaction tubes and the solution evaporated to dryness using a heating block at 100° and a gentle current of dry air. The residues were carried through the complete derivatization procedure and 2-μl aliquots of the final ethyl acetate solution were gas chromatographed. Peak areas against an internal standard (*n*-propyl, N-acetyl norleucine) added just prior to injection were calculated automatically by the PEP-2 data processor and the results analyzed statistically. Results for some amino acids are given in Table I.

TABLE I

REPRODUCIBILITY OF DERIVATIZATION

Fifteen aliquots of aqueous amino acids, each at a level of 8 μM, were derivatized without pretreatment. Levels were calculated against those from an additional analysis used as an 8-μM standard.

	Amino acid							
	Val	*Ile*	*Thr*	*Asp*	*Phe*	*Tyr*	*Trp*	*His*
Mean ($\bar{X}$)	8.06	7.99	8.12	8.00	8.05	8.01	7.98	7.85
Standard deviation (SD)	0.17	0.18	0.20	0.12	0.16	0.40	0.19	0.40
Coefficient of variation (CV), %	2.1	2.2	2.5	1.5	2.0	5.0	2.4	5.1

Derivatization efficiency was studied by derivatizing different levels of pure amino acid solutions adding an injection standard immediately prior to analysis and comparing peak areas with corresponding areas of pure distilled derivatives. Results obtained for eight replicate analyses are given in Table II. Most amino acids derivatize over a 0.1-μg to 10-μg range with at least 96% efficiency. An anomaly is arginine, which derivatizes at about 78% efficiency. This is due to the incomplete removal of HCl from the guanido group. Acylation of the *n*-propyl arginine may be improved by repeating the acylation procedure. However, the standard procedure is highly reproducible (the coefficient of variation is about 2.5%) and, provided this small loss of sensitivity for arginine is acceptable, little purpose is served by increasing the total time of analysis.

Pretreatment recoveries

Recoveries were determined using the resin pretreatment procedure. Eight separate 0.1-ml aliquots of a standard amino acid mixture, containing 1 μg of each

TABLE II

DERIVATIZATION DATA FOR AMINO ACIDS

Aqueous amino acid solutions were evaporated to dryness and derivatized. Peak areas are expressed as % of peak areas obtained with pure distilled derivatives. N = Number of samples.

	Amino acid						
	Val	*Ile*	*Thr*	*Phe*	*Lys*	*Arg*	*Trp*
Level 0.1 μg, $N=6$							
$\bar{X}$	98.7	99.3	98.7	98.8	98.3	79.0	99.8
SD	1.6	2.3	1.4	1.9	2.1	1.8	2.4
CV, %	1.7	2.3	1.5	1.9	2.1	2.3	2.4
Level 1 μg, $N=6$							
$\bar{X}$	98.0	97.7	98.1	98.7	99.9	77.9	99.1
SD	0.8	1.1	1.7	2.2	1.6	1.8	2.5
CV, %	0.8	1.1	1.7	2.2	1.6	2.3	2.5
Level 10 μg, $N=6$							
$\bar{X}$	97.8	96.8	97.5	97.6	97.5	77.8	97.6
SD	0.9	0.9	1.0	0.8	1.7	1.8	1.3
CV, %	0.9	1.0	1.1	0.8	1.8	2.3	2.4

amino acid, were adjusted to pH 2.2, processed through the resin, subsequently derivatized and gas chromatographed. Immediately prior to the injection a known amount of pure norleucine derivative was added as injection standard. Peak areas were computed and the peak area of each amino acid expressed as a percentage of the corresponding chromatographic area of the amino acid derivatized but not subjected to resin pretreatment. Results are given in Table III.

TABLE III

PRETREATMENT RECOVERIES

Aqueous solutions of amino acid subjected to the complete procedure. Pure norleucine derivative was added to processed sample as an injection standard and peak areas of each amino acid were compared with amino acids derivatized but not subjected to pretreatment. $N=8$.

	Amino acid						
	Val	*Ile*	*Ser*	*Thr*	*Asp*	*Met*	*Phe*
$\bar{X}$	0.98	0.98	0.97	0.96	0.98	0.92	0.97
SD	0.02	0.02	0.03	0.03	0.03	0.04	0.02
CV, %	2.0	2.0	3.1	2.9	2.9	4.4	2.1
	Glu	*His*	*Tyr*	*Orn*	*Lys*	*Arg*	*Trp*
$\bar{X}$	0.97	0.91	0.94	0.96	0.97	0.79	0.97
SD	0.02	0.05	0.03	0.03	0.03	0.02	0.02
CV, %	2.1	5.5	3.2	3.1	3.1	2.5	2.1

Serum recoveries

To determine recoveries from serum, where it could be expected that some absorption of amino acids by protein might occur, dialyzed serum with added amino acids was subjected to the complete procedure. Eight 0.1-ml aliquots of dialyzed serum were taken and the amino acids were added at the 1 mg/ml level. Allowance for residual amino acids was made by taking six aliquots of the serum through the procedure and computing peak area means, corresponding to residual amino acids, to provide a background correction for the recovery experiments. Results obtained from the recovery experiments were expressed as percentages of the corresponding areas of individual amino acids in runs made with amino acid solutions processed without serum. The results are given in Table IV. Recoveries are close to 100% with an excellent coefficient of variation.

TABLE IV

RECOVERIES OF AMINO ACIDS ADDED TO DIALYZED SERUM

100-μl aliquots of serum with amino acids were added at the 1-mg/dl level and subjected to the procedure. Peak areas of amino acids were compared with those of amino acids processed without serum. Values were corrected for residual amino acids in serum before additions. $N=8$.

	Amino acid						
	Val	*Ile*	*Ser*	*Thr*	*Asp*	*Met*	*Phe*
$\bar{X}$	0.96	0.99	0.97	0.97	0.99	0.93	0.96
SD	0.02	0.03	0.03	0.03	0.03	0.04	0.02
CV, %	2.1	3.0	3.1	3.1	3.0	4.3	2.1
	Glu	*His*	*Tyr*	*Orn*	*Lys*	*Arg*	*Trp*
$\bar{X}$	0.96	0.90	0.97	0.96	0.96	0.78	0.95
SD	0.03	0.05	0.04	0.03	0.05	0.02	0.02
CV, %	3.1	5.6	4.1	3.1	5.2	2.6	2.0

Physiological samples

A typical chromatogram obtained from a normal urine is illustrated in Fig. 4. The chromatogram shows the presence of most amino acids and has very little background interference indicating that the pretreatment procedure provides adequate separation of amino acids from this type of sample matrix.

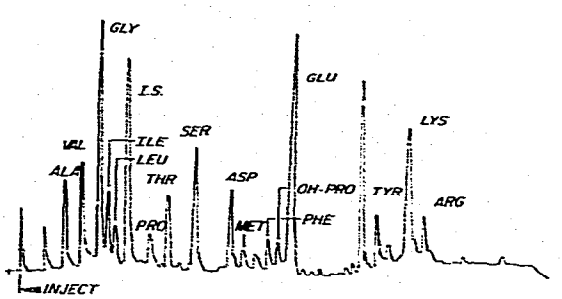

Fig. 4. Chromatogram of a normal adult urine sample subjected to the complete procedure.

The application of the procedure to a number of normal serum and urine samples was investigated. For 32 normal sera and 15 normal urines, a range of values for each amino acid was obtained. These values are given in Table V. Asparagine and glutamine are converted to their respective acids during the propylation step and these contribute to the aspartic and glutamic acid values given. The range of values corresponds reasonably well with results reported for the procedures of Stein and Moore[12] and Stein[18].

TABLE V

PHYSIOLOGICAL LEVELS OF URINARY AND SERUM AMINO ACIDS

Normal adult subjects were used for the complete procedure. $N=15$ for urines and $N=32$ for sera. Values for aspartic and glutamic acids include asparagine and glutamine.

Amino acid	*GC procedure*		*Moore and Stein procedure**		
	Urine (mg/24h) range	*Serum (mg/dl) range*	*Urine (mg/24h)*		*Serum (mg/dl) Av.*
			Range	*Av.*	
Alanine	14–65	2.9–4.3	20–70	46	3.41
Valine	2–8	2.1–4.1	—	< 10	2.88
Glycine	54–160	0.9–2.8	70–200	132	1.69
Isoleucine	12–35	0.3–1.7	10–30	18	1.54
Leucine	20–72	0.7–2.1	10–25	14	1.69
Proline	1–7	1.9–3.0	—	< 10	2.36
Threonine	18–55	1.0–1.7	15–50	28	1.39
Serine	30–70	0.7–1.9	25–75	43	1.12
Aspartic acid	14–52	0.2–1.5	—	< 10	0.03
Methionine	2–6	0.3–0.8	—	< 10	0.38
Cysteine and cystine	6–15	0.6–1.9	10–20	10	1.18
Phenylalanine	5–34	0.3–2.2	10–30	18	0.84
Hydroxyproline	2–12	1.0–0.8	—	—	—
Glutamic acid	20–80	4.8–12.9	—	< 10	0.70
Histidine	80–225	0.6–1.8	110–320	216	1.15
Tyrosine	10–42	0.6–1.5	15–50	35	1.03
Ornithine	1–4	0.1–1.2	—	< 10	0.72
Lysine	8–36	1.9–4.1	10–50	19	2.72
Arginine	1–6	0.9–2.7	—	< 10	1.51
Tryptophan	0–4	0.5–2.4	—	—	1.11

* Values reprinted from R. H. S. Thompson and I. D. P. Wootton (Editors), *Biochemical Disorders in Human Disease*, Academic Press, New York, 3rd ed., 1970.

Urine specimens from cystinuric subjects were obtained and analyzed. A chromatogram, including the relevant portion of the data processor report, of one of these is shown in Fig. 5. Compared with the normal, there is a dramatic increase in excretion of lysine, ornithine and arginine. It should be noted that automatic data reduction greatly simplifies screening procedures. The computer in this example has been instructed to suppress normal levels of amino acids from the report, printing out those that are abnormal. Cystine, analyzed here as cysteine, is increased above normal. The values are given in mg/24 h.

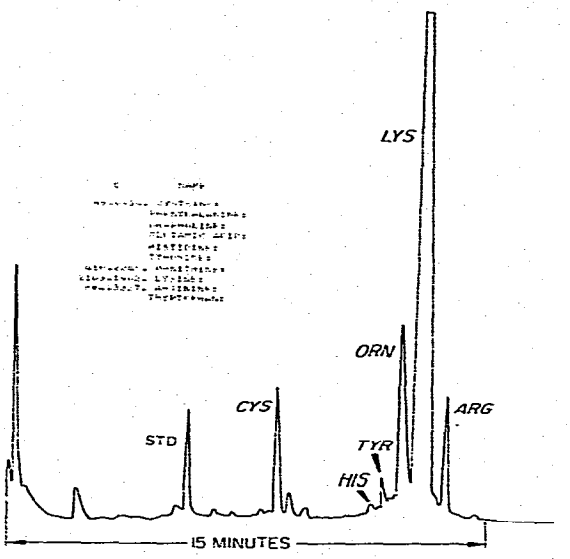

Fig. 5. Chromatogram of a cystinuric urine together with a portion of the computer report.

Several maple syrup urine samples were processed. A chromatogram obtained from a maple syrup urine is given in Fig. 6 compared with a normal urine processed identically. In the case of the maple syrup urine chromatogram, the attenuation of the GC instrument was increased to emphasize abnormal amino acid levels. In this case valine, isoleucine and leucine are clearly abnormal. The computer report for this analysis is given in Table VI compared with the report for a normal urine. The report for the abnormal urine gives only abnormalities, normal values being suppressed. In the report for the normal urine, the computer has been instructed to report all values.

Several PKU sera from both treated and untreated subjects were processed. Fig. 7 compares a chromatogram obtained from a PKU serum with that obtained from a normal serum. The level of abnormal phenylalanine was found to be 28.5

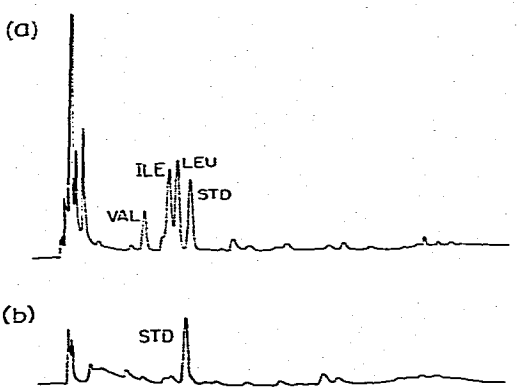

Fig. 6. Chromatogram of a maple syrup urine (a) compared with that of a normal urine (b).

TABLE VI

COMPUTER REPORTS FOR MAPLE SYRUP URINE AND NORMAL URINE SAMPLES

The normal report gives all amino acid levels. The MSU report suppresses all but abnormal values. 100 mg/dl internal standard was used for this analysis.

Run 7: Normal urine		*Run 5: MSU sample*	
C	*Name*	*C*	*Name*
40.4653,	Alanine:	.0000,	!
2.9314,	Valine:	.0000,	!
86.0321,	Glycine:	.0000,	!
16.0516,	Isoleucine:	.0000,	!
14.6568,	Leucine:	.0000,	!
100.0000,	Std:	.0000,	!
2.9931,	Proline:		Alanine:
29.9315,	Threonine:	121.3916,	Valine:
.0000,	!		Glycine:
48.0404,	Serine:	243.7520,	Isoleucine:
.0000,	!	146.9675,	Leucine:
.0000,	!	100.0000,	Std:
45.9353,	Aspartic:		Proline:
7.1788,	Methionine:		Threonine:
6.7231,	Cysteine:	.0000,	!
8.3375,	Phenylalanine:		Serine:
4.5112,	OH-Proline:		Aspartic:
34.3415,	Glutamic acid:		Methionine:
.0000,	!		Cysteine:
.0000,	!		Phenylalanine:
.0000,	!		OH-Proline:
.0000,	!		Glutamic acid:
91.0959,	Histidine:	.0000,	!
19.0444,	Tyrosine:	.0000,	!
8.1906,	Ornithine:	.0000,	!
12.2176,	Lysine:		Histidine:
5.4813,	Arginine:		Tyrosine:
	Tryptophan:		Ornithine:
			Lysine:
			Arginine:
			Tryptophan:

mg/dl. The procedure gives results that may be correlated closely with other phenylalanine procedures. It was possible to compare phenylalanine levels by the described procedures and a standard fluorimetric procedure[19] for nine identical sera. The results are compared in Table VII. The results are in excellent agreement, although in the case of the two lowest phenylalanine levels, the GC procedure gave somewhat higher values.

It is possible to use 10-μl samples of serum provided the sample is diluted to 100-μl volume with deionized water before adding the acetic acid solution. To maintain good recoveries at this level it is advisable to use micro-reaction vials such as the Reacti-vial (Pierce) in the 3-ml size for the chemical procedures and to reduce reagent volumes by half. A typical chromatogram obtained from a 10-μl PKU serum

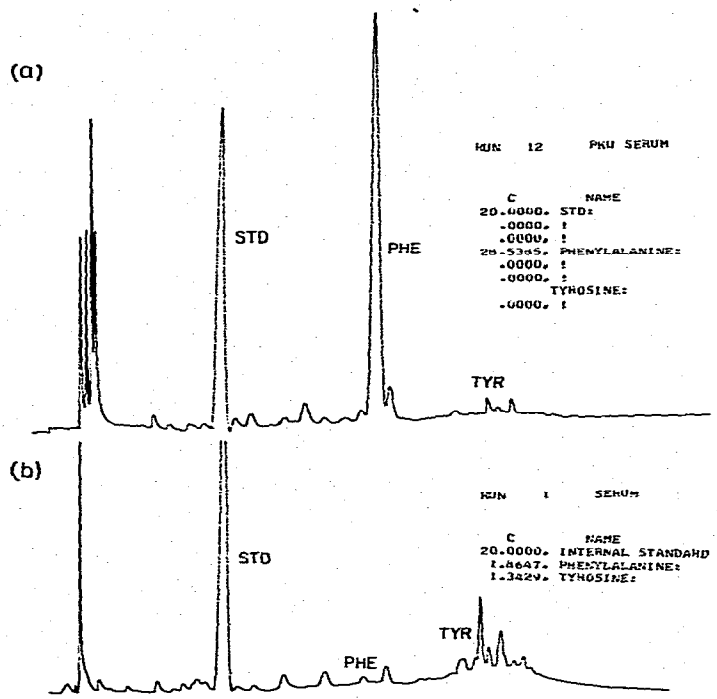

Fig. 7. Chromatogram obtained from a serum from a PKU subject (a) compared with a normal serum (b). Computer reports for each sample are included.

TABLE VII

COMPARISON OF FLUORIMETRIC AND GC METHODS

All results are the average of duplicate analyses.

Serum	*Phenylalanine (mg/dl)*	
	Fluorimetric	*GC*
1	39.5	38.7
2	25.0	25.4
3	23.0	22.8
4	17.5	16.6
5	22.5	22.7
6	13.9	13.6
7	2.0	2.4
8	2.0	2.5
9	27.6	27.2

is illustrated in Fig. 8. To maintain adequate sensitivity, the total derivatized sample was transferred to an MS-41 injection capsule, the solvent evaporated off and the GC analysis carried out. The level of phenylalanine reported was 38.7 mg/dl, a figure in close agreement with the value of 39.5 mg/dl found by the fluorimetric procedure (sample No. 1 in Table VII).

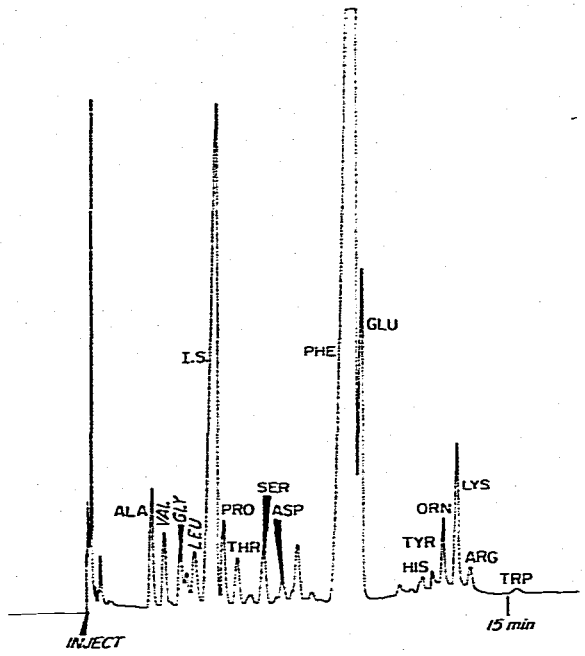

Fig. 8. Chromatogram obtained following processing of a 10-μl serum sample. The serum was obtained from a PKU subject.

Plant tissues. Several plant samples were processed in order to determine the suitability of the procedure for amino acid profiling of soluble amino acids in this type of sample. Throughout these studies, 70% ethanol extracts of the tissues were prepared following the procedure given above. A chromatogram is given in Fig. 9 from an analysis carried out on spinach leaves as representative of leaf tissue. Fig. 10 gives the chromatogram obtained from grape juice. Fig. 11 that from apple juice and Fig. 12 that from orange juice. The juices were processed without any prior extraction, the juices being centrifuged only to remove cellular debris before subjecting them to the resin column. Pigments present in the spinach and grape samples did not interfere with the analysis. Some pigment adsorbed on to the resin but did not alter its capacity to any significant degree.

Protein hydrolyzates. Protein aliquots of 1 to 10 mg were weighed into a reaction tube. 2 ml of 6 *N* HCl were added together with the 20 μg of norleucine internal standard. After flushing with nitrogen, the tube was capped and heated for 22 h at 110° ±1°. After rotary evaporation to dryness, the hydrolyzates were derivatized and gas chromatographed. A chromatogram for ribonuclease hydrolyzate prepared as above is illustrated in Fig. 13.

Six individual aliquots of ribonuclease were hydrolyzed, simultaneously processed and analyzed for amino acid content. The chromatographic data for the six runs are summarized in Table VIII and indicate the reproducibility attainable for this type of sample. No correction has been made for low recoveries of some amino acids due to the hydrolysis conditions used, the data being presented as typical of reproducibility data for the procedures.

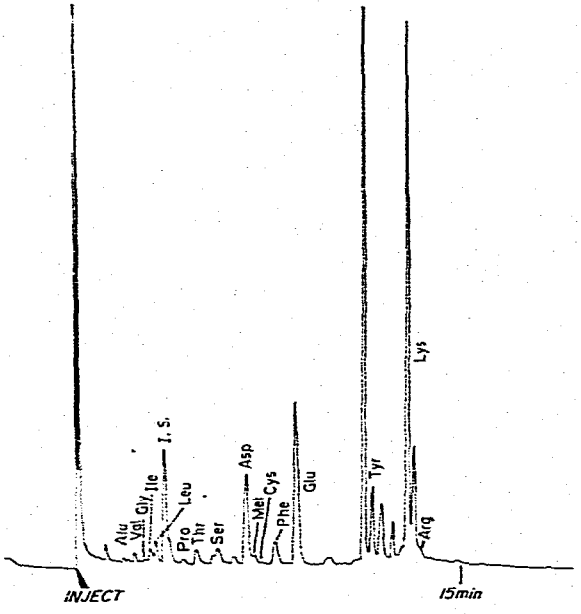

Fig. 9. Chromatogram of soluble amino acids obtained following processing of an ethanolic extract of spinach leaves. Extract equivalent to 100 mg leaf tissue was processed and 1/50 of the derivatized material was chromatographed.

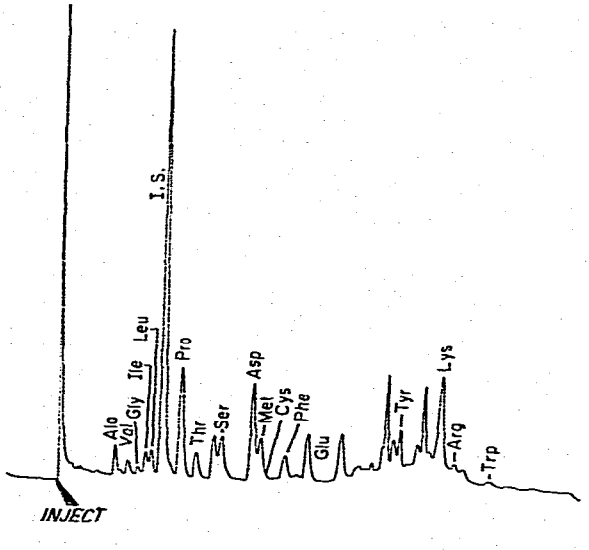

Fig. 10. Chromatogram obtained from grape juice. Injection aliquot equivalent to 20 μl of the original sample.

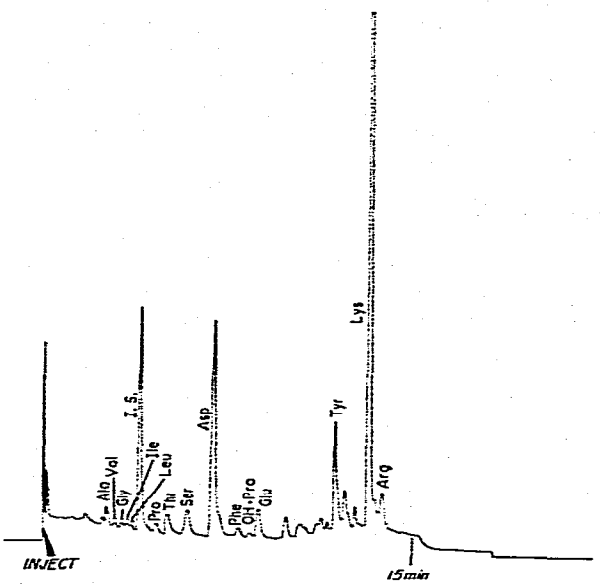

Fig. 11. Chromatogram of apple juice. Injection aliquot equivalent to 20 μl of the original sample of apple juice.

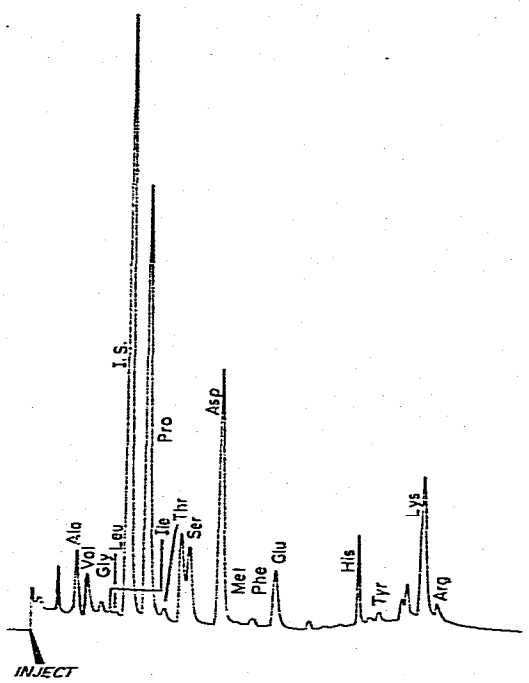

Fig. 12. Chromatogram of orange juice. Injection aliquot equivalent to 20 μl of the original juice.

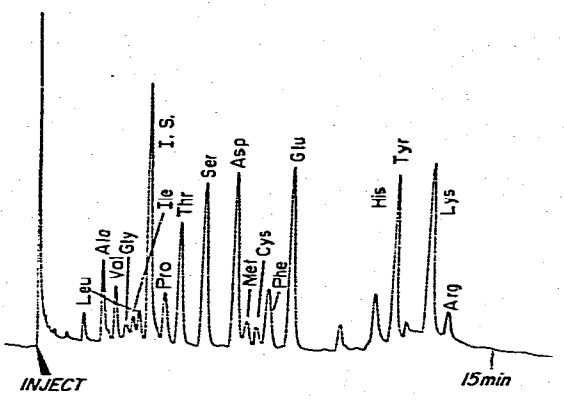

Fig. 13. Chromatogram of hydrolyzate of ribonuclease. 2 mg protein hydrolyzed, derivatized and taken up in 100 μl of ethyl acetate. 1 μl (equivalent to 20 μg protein) injected.

TABLE VIII

AMINO ACID ANALYSIS OF RIBONUCLEASE HYDROLYZATES

Reproducibility data are provided for different samples. The number of hydrolyzate samples was 6.

	$\bar{X}$	SD	CV (%)
Alanine	7.40	0.14	1.9
Valine	7.18	0.18	2.3
Glycine	1.71	0.04	2.3
Leucine	1.93	0.04	2.1
Isoleucine	1.76	0.04	2.3
Proline	3.50	0.07	2.0
Threonine	7.44	0.20	2.7
Serine	9.76	0.21	2.2
Aspartic acid	13.7	0.39	2.9
Methionine	3.53	0.09	2.6
Cysteine	4.97	0.19	3.8
Phenylalanine	3.19	0.09	2.8
Glutamic acid	12.37	0.29	2.3
Histidine	3.60	0.15	4.2
Tyrosine	6.75	0.14	2.1
Lysine	10.06	0.44	4.2
Arginine	4.16	0.14	3.4

Chromatograms for hydrolyzates of histone, ovalbumin and collagen are given in Figs. 14, 15 and 16, respectively. The amino acid content for these analyses is given in Table IX.

Stability of derivatized samples

The *n*-propyl, N-acetyl derivatives in solution are very stable under normal laboratory conditions. Stored at 4° in capped reaction tubes, the derivatized samples are stable for several weeks. Stability studies were undertaken by taking 0.1-ml

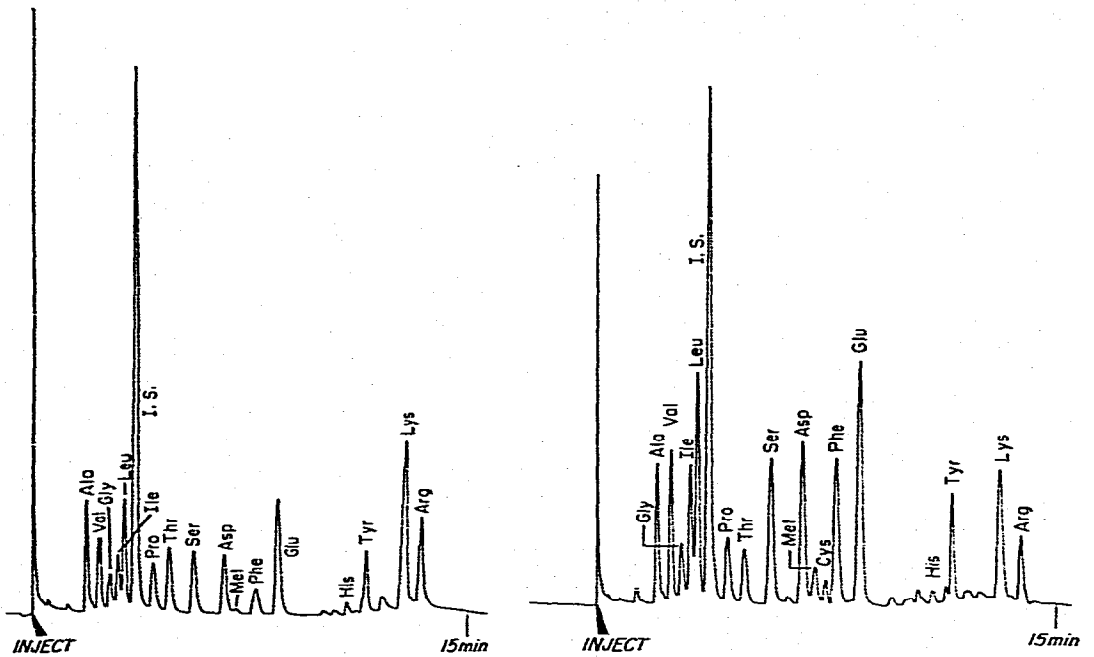

Fig. 14. Chromatogram of histone hydrolyzate. Injection aliquot equivalent to 20 μg protein.

Fig. 15. Chromatogram of ovalbumin hydrolyzate. Injection aliquot equal to 20 μg protein.

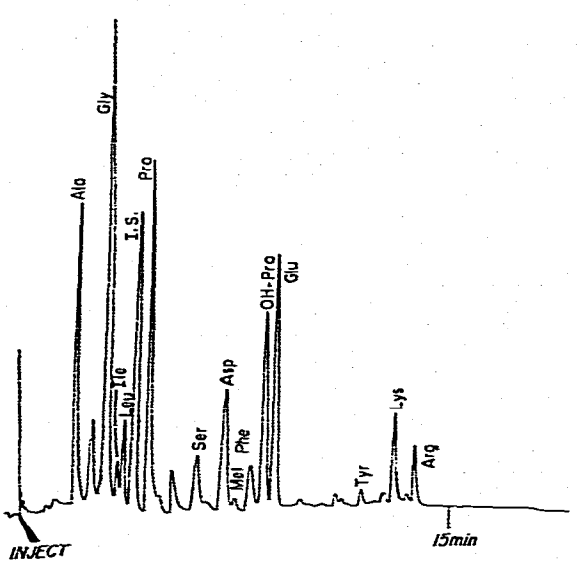

Fig. 16. Chromatogram of 20 μg derivatized collagen hydrolyzate. The hydroxyproline derivative resolves adequately from the phenylalanine and glutamic acid peaks.

TABLE IX

DATA OBTAINED FROM THE ANALYSES OF PROTEIN HYDROLYZATES

No correction has been made for losses due to the hydrolysis conditions used. The results are the average of duplicate analyses.

Amino acid	*g amino acid/100 g protein*		
	Collagen	*Histone*	*Ovalbumin*
Alanine	9.3	8.6	2.2
Valine	2.1	7.4	3.2
Glycine	25.6	5.6	1.2
Isoleucine	1.0	3.4	3.3
Leucine	2.8	6.5	0.4
Proline	11.9	4.2	1.5
Threonine	1.8	6.0	1.2
Serine	3.8	4.3	3.1
Aspartic acid	5.4	4.0	4.0
Methionine	0.6	0.3	2.2
Phenylalanine	1.9	3.0	3.4
Hydroxyproline	13.0	0	0
Glutamic acid	11.5	10.3	7.4
Histidine	0.2	3.2	18.9
Tyrosine	0.4	1.7	15.3
Lysine	3.4	15.2	24.2
Arginine	7.9	9.1	22.1
Cysteine	0	0	0.7

aliquots of dialyzed serum. Levels of phenylalanine equal to 5, 10, 20 and 50 mg/dl were added to duplicate samples and the serum samples carried through the complete procedure. The samples were gas chromatographed immediately after processing. The samples were resealed and stored at 4° for 14 days, then gas chromatographed a second time. Results on duplicates for each time of analysis were averaged. The levels that were found are given in Table X. The results are in excellent agreement.

The results obtained for a serum analysis immediately after processing were compared with an analysis carried out on the same derivatized sample but after sample storage for 4 weeks and 8 weeks at 4°. This comparison is given in Table XI

TABLE X

STABILITY OF DERIVATIZED SERUM SAMPLES CONTAINING ADDED PHENYLALANINE

Samples were analyzed immediately after processing and after 14 days' storage at 4°.

	Added phenylalanine level (mg/dl)			
	5	*10*	*20*	*50*
Analyzed immediately after processing	4.94	10.02	19.86	48.95
Analyzed after 14 days' storage	4.87	9.97	19.94	48.20

TABLE XI

STABILITY OF DERIVATIZED SERUM SAMPLES

The values in mg/dl were obtained by chromatographing immediately on processing and after 4 and 8 weeks' storage at 4°

Amino acid	*Storage time (weeks)*		
	0	*4*	*8*
Alanine	3.7	3.5	3.6
Glycine	1.1	1.2	1.2
Leucine	1.8	1.7	1.8
Proline	2.2	2.4	2.2
Threonine	1.3	1.2	1.2
Cysteine	0.9	1.0	1.0
Phenylalanine	0.8	0.8	0.8
Hydroxyproline	0.4	0.4	0.5
Histidine	1.2	0.9	0.8
Lysine	2.3	2.4	2.4
Arginine	1.9	1.8	1.8
Tryptophan	1.8	1.9	1.8

for several of the amino acids. The histidine level is considerably lower for the stored sample. The chromatograms showed an extra peak between ornithine and lysine. By adding small amounts of water to derivatized histidine, it was subsequently found that *n*-propyl diacetyl histidine is partially deacylated to the monoacetyl ester in the presence of moisture. The monoacetyl ester chromatographs between the ornithine and lysine derivatives. The deacylation is largely eliminated by use of anhydrous ethyl acetate in taking up the final residue. Also the histidine derivative is most stable in solution, the dry residue following derivatization apparently being more sensitive to traces of moisture. Immediate solubilization of the residue in anhydrous ethyl acetate is therefore essential if stability of the histidine derivative is a prime consideration.

DISCUSSION AND CONCLUSION

The described procedures offer a convenient addition to other methods for routine amino acid analysis. Without imposing too great a demand on operator skill or demanding sophisticated equipment, the procedures are capable of producing excellent reproducible results on a wide range of samples. The derivatives have the advantage of being highly stable under average laboratory conditions. Overall analysis time for a single sample using the complete procedure is about 1 h. Considerable savings in time may be made by processing several samples simultaneously. GC does not, of course, provide positive identification for any particular peak. It does, however, in combination with a suitable sample pretreatment procedure, provide for considerable reliability in identification by using retention time characteristics. The pretreatment step using cation-exchange resin with a high cross-linkage is effective in removing anions, large molecules and non-ionic material such as sugars, lipids, polypeptides and protein from the sample. However, it would

be expected that amines and some small peptides would be present in the final material. It is clear from the results that these, for the samples investigated, do not pose a serious problem. It is to be anticipated that some peptide material would derivatize and have retention characteristics similar to those of some of the common amino acids and could therefore contribute to the level reported for an amino acid. In the case of sera and urine analyses, it would be expected that the wide difference between normal and abnormal values would lead to no confusion of interpretation. Indeed, this has been the experience of this worker.

A possible source of interference resides in the less common amino acids. To determine the retention characteristics of several of these, samples of the pure amino acids were processed and chromatographed. The relative retention time of each compared with neighboring amino acids is given in Table XII. Several of these are of interest as they do occur and may be identified in biological samples. Asparagine and glutamine will, when these procedures are used, be analyzed as aspartic and glutamic acids. Citrulline is not analyzed by these procedures. The resin pretreatment step loses taurine and cysteic acid in the effluent.

TABLE XII

RRT CHARACTERISTICS OF SOME COMPOUNDS THAT MAY BE PROCESSED WITH THE COMMON AMINO ACIDS

The RRT is measured relative to norleucine = 1.000.

Compound	*RRT*	
α-Aminoisobutyric acid	0.524	
	0.547	Alanine
β-Amino-*n*-butyric acid	0.827	
	0.837	Isoleucine
β-Aminoisobutyric acid	0.887	
γ-Amino-*n*-butyric acid	1.494	
	1.510	Serine
Asparagine	1.823	Aspartic acid
ε-Amino-*n*-caproic acid	2.106	
	2.154	Phenylalanine
Glutamine	2.290	Glutamic acid
Aminoadipic acid	2.383	
α-Aminopimelic acid	2.773	
	3.034	Histidine

The sensitivity of flame ionization detectors used in GC analysis provides the capability of quantitating levels of a pure compound down to 10^{-12} mole. In practice, the sensitivity attainable is dependent on factors including column performance, background of the sample, and the molar response of the particular amino acid to be quantitated. The results obtained for the sera samples using 100-μl sample size and taking 10-μl aliquots for the GC analyses indicate that, for those amino acids with excellent detector response such as hydroxyproline, it is readily possible to quantitate the equivalent of 50 ng of free amino acid.

ACKNOWLEDGEMENTS

My thanks are due to Ms. Phyllis Taylor (State Department of Health, Hartford, Conn.), Dr. John Meola (Albany Medical Center, Albany, N.Y.), and Graff Associates (Santa Clara, Calif.) for generous provision of abnormal clinical samples. John Meola carried out the fluorimetric procedures for many of the serum phenylalanine levels used in this report. Mr. Gary Schmidt provided able technical assistance. I thank W. Slavin for constructive advice with this manuscript.

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