methylpyrazines and reactions of fragments II and III with acetaldehyde to form fragments VIII and X, respectively, which subsequently produce larger alkylpyrazines (Figure 1). Fragments II and III, therefore, must react with fragments I, V, or VI to form methyl- or dimethylpyrazines before being condensed with acetaldehyde to give fragments VIII or X.

There is a greater yield of unsubstituted pyrazines from the glyceraldehyde system than from the dihydroxyacetone system. This may well depend upon the ease of formation of fragment I which can form directly from glyceraldehyde.

Stereochemical Problem of Dehydration Reaction. It is assumed that fragments II and V are essential fragments for formation of 2,6-dimethylpyrazine. In any case, fragment V should form as long as fragment I forms, and the facile formation of fragment I is obvious because of the high yield of 2-methylpyrazine. Therefore, diminished production of fragment II causes this difference in yield.

One of the differences between glucose and galactose, and xylose and arabinose is that the hydroxy groups on carbons 3 and 4 are in the trans and cis configurations, respectively. If there is a possibility of neighboring group participation in these dehydration reactions in addition to a β-elimination reaction, the trans form must be favored to eliminate water (Cram, 1952). On the other hand, these differences may be due simply to the different reactivity of the sugars. Further investigation is necessary to clarify this point.

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Gas-Liquid Chromatographic Analysis of Amino Acids in Food Samples

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The gas chromatographic method of amino acid analysis requires that the sample is hydrolyzed to release the amino acids, which are then cleaned in an ion-exchange column, converted to volatile derivatives, and then separated on a polyester as well as on a silicone column. These different steps contribute to certain amounts of variation in the recovery of the individual amino acids. In addition, the various components of the food samples, especially carbohydrates, could affect the recovery of some amino acids. In this paper certain quantitative aspects of amino acid analysis of food samples by gas chromatography are investigated. The recovery of amino acids as affected by duration of hydrolysis and presence of various carbohydrates during hydrolysis is estimated. Furthermore, the amino acid patterns determined by GLC of a series of diet samples collected under a nutrition survey program are compared with the values obtained by ion-exchange chromatography.

Proteins are one of the most important dietary components of human food. The nutritive value of the protein depends mainly on its amino acid composition. As a result of the scarcity of proteins and increasing consciousness of the aspects of protein nutrition and health, there is a great demand for a routine method for amino acid analysis for the evaluation of the proteins in raw materials and estimation of the effect of various steps of manufacturing on the nutritional quality of protein in the finished product.

Ion-exchange chromatography (Moore and Stein, 1951) is so far the most widely used method of amino acid analysis. Nevertheless, due to speed, sensitivity, and versatility, gas chromatography could be a cheaper alternative. The experimental conditions for the quantitative derivatization and chromatographic requirements for their separation are detailed in a series of publications

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by Gehrke and his co-workers. The amino acids, converted to *N*-trifluoroacetyl-*n*-butyl derivatives, are eluted quantitatively from two separate columns containing 0.65 w/w % EGA on Chromosorb W and 1.5 w/w % OV-17 on Chromosorb G. The OV-17 column was used to separate arginine, histidine, and cystine. Instead of the OV-17 column, a mixed phase column containing 2 w/w % OV-17 and 1 w/w % OV-210 coated on 100/120 mesh Supelcoport could also be used (Gehrke et al., 1971; Kaiser et al., 1974).

Before making derivatives of the amino acids and further GLC separation, the amino acids have to be released from the proteins by a suitable method of hydrolysis. Heating the protein sample with an adequate quantity of 6 N HCl at about 110 °C for a period of 20–24 h is the most commonly used method. The majority of the amino acids are liberated completely from the proteins during the acid hydrolysis and are stable under the above conditions of hydrolysis. However, often in the amino acid analysis, the hydrolysis step is perhaps the important cause of variability (Hill, 1965). An exhaustive review of literature concerning protein hydrolysis and its effects on the recovery of different amino acids is given by Roach and Gehrke (1970). The extent to which the hydrolysis can be completed and the stability of the released amino acids in the hydrolytic medium depend, among other factors, on the composition of the biological sample and the structure of the protein molecules. The hydrolytic reagent might react more or less intensively on different parts of the molecule depending not only on their molecular structure but also on the steric hindrance due to bulky side chains of aliphatic amino acids.

Sample components like starch, sugar, metal ions, oxygen, or free halogens would also affect the stability of the released amino acids. Recovery of amino acids from wheat bran after acid hydrolysis showed no increase or decrease after 24 h at 110 °C, though determination of separate correction factors was necessary for serine and threonine, which are released readily but are not stable, and for valine and isoleucine, which are stable but not easily released (Kohler and Plater, 1967). In addition to the above mentioned, there are a few other amino acids which show major losses during acid hydrolysis, namely cysteine, cystine, methionine, tryptophan, and tyrosine (Finlayson, 1965). Tryptophan can be protected from destruction during acid hydrolysis with the addition of 2% thioglycolic acid in 6 N HCl, when the sample is free from carbohydrates (Matsubara and Sasaki, 1969). The current method of complete amino acid analysis requires acid hydrolysis by heating the sample with 6 N HCl for 20–24 h in the absence of oxygen, performic acid treatment before acid hydrolysis, and basic hydrolysis with 8 N barium hydroxide.

In the present work we report the results of some experiments conducted to investigate certain quantitative aspects of acid hydrolysis, ion-exchange cleanup of the free amino acids, derivatization to *N*-trifluoroacetyl-*n*-butyl derivatives, and their gas chromatographic separation. The recovery of amino acids from a sample of human serum albumin hydrolyzed at various intervals is studied. The effect of different carbohydrates present in the sample on recovery after acid hydrolysis is investigated. The GLC method is applied to analyze a number of diet samples from a nutrition survey, and these values are compared with the values obtained by an ion-exchange chromatographic method.

MATERIALS AND METHODS

Diet Samples. Fat-extracted and lyophilized 1-day specimens of mixed food from a duplicate portion nutrition survey (Borgström et al., 1975) were used.

Chemicals. All the standard amino acids were from British Drug House (BDH), Poole, England. Isoleucine (alloisoleucine free) was bought from Calbiochem AG, Lucerne, Switzerland. α-Aminocaprylic acid was from Sigma Chemical Co., St. Louis, Mo. Hydrochloric acid (Aristar) used for hydrolysis, butanol for esterification, and strong cation-exchange resin Amberlite-IR-120 H were from BDH, Poole, England. The hydrochloric acid gas was from Matheson & Co. Trifluoroacetic anhydride was an Eastman Kodak product. Methylene chloride was from Merck.

Proteins and Carbohydrates. Human serum albumin was from Kabi AB, Stockholm, Sweden. Fructose, galactose, lactose, sucrose, and soluble starch were from Merck AG, Darmstadt, West Germany. Cellulose (CEPO) was from the Swedish Cellulose Powder & Woodflour Mills Ltd., Gothenburg, Sweden.

Hydrolysis. The samples were weighed into a hydrolysis tube (16 × 160 mm Duran 50 test tubes). Five milliliters of 6 N HCl containing 10 g of phenol per liter was added to the tubes. After adding the adequate amount of internal standard solution (α-aminocaprylic acid), the tubes were kept frozen in a mixture of solid CO_2 and ethanol. The frozen samples were placed in a desiccator. By applying vacuum for a period of 20 min, the dissolved air and O_2 were removed. Dry N_2 was let in to release the vacuum. The tubes were then sealed and kept constantly rotating in a hot-air oven under 110 °C for 24 h. For the analysis of human serum albumin, a solution of it in 6 N HCl (0.5 mg/mL) was made. Five milliliters of this solution was taken into the hydrolysis tubes, along with internal standard for hydrolysis.

Ion-Exchange Cleanup. To remove the impurities which might interfere with the GLC separation, it was necessary to clean the hydrolysate through an ion-exchange resin column. Amberlite-IR-120, a strong cation-exchange resin in hydrogen form, was treated as follows before filling it into the columns. The resin was washed with 7 N NH_4OH by stirring it with a magnetic stirrer for 1 h. This was repeated a couple of times before the resin was washed with deionized water until it was neutral to litmus paper, and then an excess of 3 N HCl was added and stirred over a magnetic stirrer very slowly. After 1 h, the HCl was poured away and the resin was washed with deionized water to neutral pH. The resin treated as above was filled into the columns carefully without pockets and air bubbles. The hydrolysate or the amino acid mixture was evaporated first and then dissolved in 0.1 M HCl. This solution was placed over the ion-exchange column without disturbing the resin bed. The impurities were washed out by passing 5 mL of deionized water through the column. The amino acids were then eluted with 5 mL of 7 N NH_4OH, followed by 5 mL of deionized water. The column was regenerated with 3 N HCl before further use.

Derivatization. An aliquot of the hydrolysate or standard amino acid solution was transferred into small acylation tubes with bakelite caps having Teflon packing. The tubes were placed on a sand bath and evaporated to dryness by blowing dry nitrogen into them. To the dry sample 3 N HCl–butanol was added and the closed tubes were kept at 100 °C for 30 min for esterification. When the esterification was complete, the samples were dried as above and acylated at 100 °C for 30 min in closed tubes with trifluoroacetic anhydride using methylene chloride as solvent.

Gas Chromatographic Analysis. A PYE Unicam 140 gas chromatograph with a flame ionization detector and

Table I. Amino Acid Analysis of Human Serum Albumin by Gas–Liquid Chromatography and by Ion–Exchange Chromatography

	w/w, %					
Amino acids	GLC	Diff,[a] % (1 – 2)	CIE	Diff,[a] % (1 – 3)	Lit.[b]	Diff,[a] % (2 – 3)
Alanine	6.25	2.0	6.42	21.6	7.6	18.3
Valine	5.69	6.5	5.34	3.7	5.9	10.4
Glycine	1.35	19.4	1.13	12.5	1.2	6.2
Isoleucine	1.12	4.4	1.17	42.8	1.6	36.0
Leucine	10.20	10.1	9.26	4.9	10.7	15.5
Proline	3.78	10.5	3.42	11.2	3.4	0.5
Threonine	3.56	8.9	3.88	13.7	4.3	10.8
Serine	3.11	10.7	2.81	2.9	3.2	13.8
Phenylalanine	5.37	9.3	5.87	22.9	6.6	12.4
Aspartic acid	9.54	12.7	8.46	0.6	9.6	13.4
Glutamic acid	15.99	10.4	14.32	2.6	16.4	14.5
Tyrosine	3.90	0.5	3.88	0.0	3.9	0.5
Lysine	11.19	11.2	10.06	6.6	10.5	4.3
Mean difference		8.96		11.03		12.0

[a] 1, GLC; 2, ion-exchange chromatography; 3, literature (Kirschenbaum, 1972). [b] w/w % was calculated from number of residues per mole assuming molecular weight as 65 000.

temperature program facility was used. The detector signals were integrated using a Vidar Autolab 6300 electronic digital integrator, and the chromatograms were recorded on a Hitachi Perkin-Elmer Qa 15 recorder. All the GLC separations were done on glass columns (1700 × 4 mm) containing 0.65 w/w % stabilized ethylene glycol adipate on high-performance Chromosorb W 80/100 mesh which was acid washed and DMCS treated. The columns were conditioned overnight at 230 °C with an argon flow rate of 50 mL/min through them.

Ion-exchange chromotographic analysis was done on an amino acid analyzer (Biocal 2000 autoanalyzer) using norleucine as internal standard.

RESULTS AND DISCUSSION

Accuracy of the Amino Acid Analysis by Gas Chromatography. A sample of human serum albumin was hydrolyzed as described previously, and the hydrolysate was divided into two equal portions. One portion was analyzed for amino acid content by ion-exchange chromatography and the other half was analyzed by gas chromatography (Figure 1). The results are shown in Table I along with the values calculated from the residue data available in the literature. The amino acid composition of human serum albumin in the literature was given as number of residues per molecule and percentages were calculated assuming that the molecular weight was 65 000. The analysis by ion-exchange chromatography was done using norleucine as internal standard and α-aminocaprylic acid was used in the gas chromatographic analysis. It is obvious that the results of analysis by the two methods agree well and the difference between the values in the literature and each of the methods is higher than the difference between the methods. The greatest difference was between glycine, 19.4%, and the least difference was between tyrosine, 0.5%. The mean difference for all the amino acids together was only 8.96%. The mean difference of all the amino acids between that determined by ion-exchange chromatography and the literature value is 12.0% and that between GLC and the literature value is 11.03%. The sulfur amino acids and tryptophan were not determined by GLC.

Precision of Amino Acid Analysis by GLC. To determine the precision of the method, four separate samples of human serum albumin were hydrolyzed separately and carried through the procedure for determination of the amino acid content. The mean values and the respective standard deviations along with the relative standard deviation percentages are given in Table II. Strictly, many amino acids are not stable during acid hydrolysis, and we have in this study included those amino acids which can be considered as being released quanti-

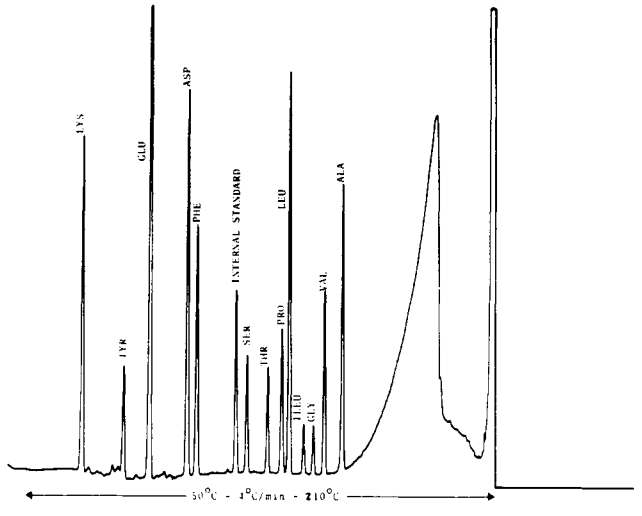

Figure 1. Separation of amino acids from human serum albumin as their *N*-trifluoroacetyl-*n*-butyl (TAB) derivation on an EGA column (1500 mm × 4 mm glass column, 0.65 w/w % EGA on 80/100 mesh Chromosorb W HP, carrier gas (argon) 50 mL/min, hydrogen 30 mL/min, air 300 mL/min).

Table II. Precision of Amino Acid Analysis by GLC of a Sample of Human Serum Albumin

Amino acid	w/w, %, mean	SD	SE	Rel SD, %
Alanine	6.25	0.567	0.321	9.07
Valine	5.69	0.619	0.384	10.88
Glycine	1.15	0.108	0.015	11.16
Isoleucine	1.15	0.128	0.016	11.16
Leucine	10.03	0.281	0.140	2.80
Proline	3.85	0.153	0.023	3.97
Threonine	3.58	0.318	0.159	8.89
Serine	3.11	0.195	0.097	6.27
Phenylalanine	5.63	0.134	0.157	5.58
Aspartic acid	9.54	0.475	0.237	4.98
Glutamic acid	15.99	0.796	0.398	4.98
Tyrosine	3.90	0.153	0.076	3.92
Lysine	11.20	0.596	0.298	5.32
Mean rel SD				6.85

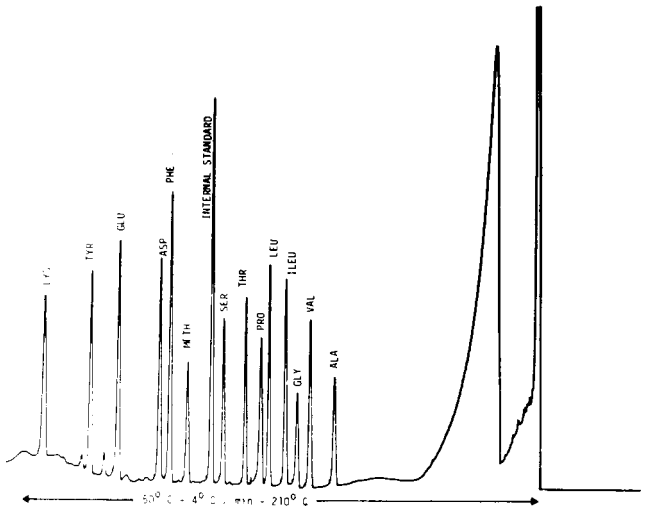

Figure 2. Separation of a standard mixture of amino acids as their TAB derivatives on an EGA column (for chromatographic conditions see Figure 1).

Table III. Precision of the Ion-Exchange Cleanup, Derivatization, and GLC Analysis of a Standard Amino Acid Mixture

Amino acid	A_{aa}/A_{is},[a] mean	SE	SD	Rel SD, %
Alanine	0.428	0.025	0.051	0.25
Valine	0.691	0.007	0.014	2.03
Glycine	0.370	0.009	0.018	4.86
Isoleucine	0.787	0.012	0.024	3.05
Leucine	0.808	0.009	0.018	2.23
Proline	0.686	0.013	0.026	3.79
Threonine	0.619	0.004	0.008	1.13
Serine	0.516	0.006	0.012	2.76
Phenylalanine	1.006	0.036	0.072	7.16
Aspartic acid	0.827	0.028	0.056	6.76
Glutamic acid	0.903	0.008	0.016	1.77
Tyrosine	0.548	0.036	0.067	12.23
Lysine	0.992	0.032	0.063	6.63
Mean rel SD				4.18

[a] A_{aa}/A_{is}, peak area of amino acid in relation to peak area of internal standard.

tatively and not destroyed by hydrolytic conditions employed. As is clear from Table II, all the amino acids showed good precision in their recovery. The lowest relative standard deviation was for leucine and highest for glycine and isoleucine. The average relative standard deviation percentage for all the amino acids together was 6.85%. This shows that the amino acid analysis as far as these amino acids are concerned can be done with satisfactory precision.

Precision of Ion-Exchange Cleanup Chromatography, Derivatization, and Gas Chromatographic Run. For the determination of the amount of precision in the various steps mentioned above, a standard mixture of amino acids was analyzed (Figure 2). An aliquot of the solution was placed in the cation-exchange column and then released with 7 N NH_4OH after washing with water. To the eluate an appropriate amount of internal standard (α-aminocaprylic acid) was added, derivatized, and then injected into the gas chromatograph. One of the four samples was divided into four parts of equal amounts and derivatized. One of the derivatized samples was injected into the gas chromatograph four times consecutively. The mean values, standard deviation, standard error of the mean, and relative standard deviation in percent of the mean are presented in Tables III, IV, and V. The mean deviation for all the amino acids analyzed in ion-exchange

Table IV. Precision of the Derivatization and GLC Analysis of a Standard Amino Acid Mixture

Amino acid	A_{aa}/A_{is},[a] mean	SE	SD	Rel SD, %
Alanine	0.482	0.003	0.007	1.45
Valine	0.675	0.011	0.022	3.26
Glycine	0.368	0.004	0.008	2.17
Isoleucine	0.792	0.017	0.035	4.42
Leucine	0.816	0.014	0.028	3.43
Proline	0.703	0.024	0.049	6.00
Threonine	0.627	0.008	0.017	2.71
Serine	0.519	0.013	0.027	5.20
Phenylalanine	1.134	0.002	0.004	0.35
Aspartic acid	0.859	0.026	0.053	6.17
Glutamic acid	0.977	0.006	0.012	1.23
Tyrosine	0.655	0.007	0.015	2.29
Lysine	1.059	1.019	0.038	3.59
Mean rel SD				3.25

[a] A_{aa}/A_{is}, peak area of amino acid in relation to peak area of internal standard.

Table V. Precision of the GLC Analysis of *N*-Trifluoroacetyl-*n*-butyl Derivatives of a Standard Amino Acid Mixture

Amino acid	A_{aa}/A_{is},[a] mean	SE	SD	Rel SD, %
Alanine	0.476	0.008	0.016	3.36
Valine	0.692	0.004	0.009	1.30
Glycine	0.363	0.009	0.018	4.96
Isoleucine	0.792	0.006	0.014	1.77
Leucine	0.816	0.002	0.004	0.49
Proline	0.696	0.006	0.013	1.87
Threonine	0.626	0.005	0.010	1.60
Serine	0.512	0.003	0.069	1.34
Phenylalanine	1.101	0.005	0.011	1.00
Aspartic acid	0.843	0.006	0.013	1.54
Glutamic acid	0.946	0.007	0.014	1.48
Tyrosine	0.662	0.015	0.030	4.53
Lysine	0.934	0.032	0.064	6.85
Mean rel SD				2.46

[a] A_{aa}/A_{is}, peak area of amino acid in relation to peak area of internal standard.

cleanup, derivatization, and GLC was 4.18%, while for derivatization and GLC it was 3.25% and for GLC alone, 2.46%. The relative standard deviation for derivatization alone was 2.12% and for ion-exchange cleanup, 0.92%. It appears that any improvement in the precision of the method would require repetition in hydrolysis rather than ion-exchange cleanup, derivatization, or GLC. Also, with the ion-exchange chromatographic method of amino acid analysis, the mean relative standard deviation calculated from a number of published results of amino acid analysis values, about 8% ranging from 5.9 to 14.1%, was observed between samples (Kwolek and Canvins, 1971). The relative standard deviation for the amino acids of different aliquots of the same hydrolysate was between 1.7 and 3.7%. Variation between different hydrolysates was 1.1% (isoleucine) to 25.2% (serine), giving mean relative standard deviation for all the amino acids between 1.9 and 12.8%. A major source of error in amino acid analysis arises from sampling, and increased precision could probably be achieved by increasing the number of samples for hydrolysis (Knippel et al., 1971).

Effect of Duration of Hydrolysis on the Recovery of Amino Acids. Often in the amino acid analysis, the hydrolysis step is the important cause for variability. The macromolecular structure and the steric hindrance offered by side chains of aliphatic amino acids cause the activity of the hydrolytic reagent at various regions of the protein. Moreover, the components other than protein would also

Table VI. Recovery of Amino Acids from a Sample of Human Serum Albumin Hydrolyzed under Different Periods at 110 °C[a]

Amino acid	Hours						
	5	10	18	24	48	72	120
Alanine	7.50	7.77	7.88	8.45	7.75	7.58	7.18
Valine	4.63	5.38	5.58	5.70	6.78	7.50	7.35
Glycine	1.18	1.15	1.27	1.28	1.20	1.13	1.20
Isoleucine	0.73	1.30	1.78	1.80	1.93	1.98	1.99
Leucine	10.20	11.20	11.21	11.33	11.65	11.57	11.38
Proline	3.50	3.70	3.88	4.03	4.13	3.88	3.98
Threonine	3.48	3.53	3.48	3.30	2.53	2.55	1.65
Serine	3.33	3.95	3.88	3.85	3.40	1.40	1.85
Phenylalanine	4.23	4.80	4.85	5.03	4.85	5.25	5.43
Aspartic acid	9.58	10.08	9.78	9.93	9.53	9.73	10.95
Glutamic acid	14.78	15.28	15.50	15.43	15.40	15.60	15.78
Tyrosine	4.43	4.73	4.73	5.25	4.43	2.78	2.93
Lysine	9.88	11.85	12.53	12.73	13.05	13.50	13.50

[a] Each value is an average of four hydrolyses of 1-mg samples (single derivatization and GLC analysis).

Table VII. Recovery of Amino Acids from a Sample of Human Serum Albumin after Acid Hydrolysis in the Presence of Various Carbohydrates[a]

Amino acid	AA/Glu, O	Glc	Gal	Fru	Lac	Suc	ST	CELL
Alanine	0.365	0.363	0.352	0.388	0.387	0.403	0.383	0.357
Valine	0.322	0.343	0.323	0.347	0.332	0.345	0.330	0.321
Glycine	0.057	0.039	0.044	0.052	0.049	0.040	0.051	0.043
Isoleucine	0.064	0.076	0.067	0.072	0.065	0.072	0.064	0.067
Leucine	0.585	0.624	0.578	0.622	0.575	0.601	0.597	0.589
Proline	0.203	0.194	0.193	0.194	0.195	0.206	0.200	0.192
Threonine	0.196	0.198	0.169	0.220	0.197	0.187	0.196	0.190
Serine	0.140	0.141	0.123	0.152	0.146	0.130	0.143	0.136
Phenylalanine	0.413	0.394	0.376	0.343	0.325	0.358	0.373	0.354
Aspartic acid	0.570	0.595	0.570	0.580	0.594	0.562	0.581	0.574
Glutamic acid	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Tyrosine	0.163	0.107	0.055	0.074	0.067	0.097	0.063	0.065
Lysine	0.623	0.630	0.500	0.691	0.543	0.616	0.664	0.617

[a] Each value is a mean of four determinations. Each carbohydrate constitutes 80% of the sample: O, without carbohydrate; Glc, glucose; Gal, galactose; Fru, fructose; Lac, lactose; Suc, sucrose; ST, starch; CELL, cellulose.

affect the recovery of amino acids. We have studied the extent to which hydrolysis could be completed and the stability of the released amino acids in the hydrolytic medium using human serum albumin. Hydrolysis was done as described in the Materials and Methods section. Four samples were taken out at 5, 10, 18, 24, 48, 72, and 120 h, respectively, for analysis. The results are shown in Table VI. For tyrosine, serine, threonine, and alanine, there were obvious losses after prolonged hydrolysis, with the highest yield obtained after 24 h of hydrolysis. The other amino acids were fairly stable even after 120 h. Over 90% of each amino acid, except for valine, had been released after 24 h of hydrolysis.

Finlayson (1965) has earlier reported losses of serine and threonine on prolonged acid hydrolysis.

Effect of the Presence of Various Carbohydrates with the Protein Sample during Hydrolysis on the Recovery of Amino Acids. Many sample components like starch, sugar, and metal ions and oxygen, or any other oxidizing agents like halogens would affect the stability of the released amino acids while analyzing food samples for their amino acid content (Roach and Gehrke, 1970). It is very valuable to know how the recovery of various amino acids depends on the content of other components, especially carbohydrates which are always present in food samples in large quantities. To study this we have hydrolyzed human serum albumin in the presence of various carbohydrates. The carbohydrates added were glucose, galactose, fructose, lactose, sucrose, soluble starch, and pure cellulose in a protein to carbohydrate ratio of 20:80. The results are shown in Table VII. The recovery of the amino acids was calculated assuming that of the glutamic acid as the unit. Tyrosine shows variation between 0.163, when protein is hydrolyzed alone, and 0.055, when hydrolyzed with galactose. The presence of galactose, at 80% level in the sample, seems to reduce the recovery of all the amino acids. In general, it can be concluded that the presence of common sugars, starch, and cellulose does not affect the recovery of amino acids studied when subjected to acid hydrolysis. This is in agreement with the results of Dustin et al. (1953), who found that large amounts of starch did not cause significant losses of amino acids. Robel (1973) suggests a sample to acid ratio greater than 1:700 for minimizing the destruction of amino acids by carbohydrates while hydrolyzing feeds and mixed diets. In our experiments we have used 1000 times acid to sample. Smith et al. (1965) found no reduction of amino acid recovery while adding D-ribose, but he noted lower values for proline and tyrosine. However, larger amounts of acid reduced the effect.

Analysis of 1-Day Specimens of Mixed Food Samples from a Duplicate Portion Nutrition Survey. Mixed food samples were analyzed for amino acid content mainly to evaluate the quantity and quality of daily protein intake by a group of men and women. This was carried out in connection with an extensive nutrition survey by a community health center near our institute. The purpose of the study reported here was to compare results from gas chromatographic analysis with those from ion-exchange chromatography and to determine whether we could apply the gas chromatographic method for the analysis of amino acids on a large number of samples. Table VIII and Table IX show the results of the analysis of 1-day mixed food samples collected from five men and five women on each

Table VIII. Amino Acid Composition of 1-Day Mixed Food Diet Samples: Five Women (Values Are Expressed as g/Day)[a]

	D 8		D 9		D 10		D 11		D 12	
Amino acid	GLC	IE	GLC	IE	GLC	IE	GLC	IE	GLC	IE
Alanine	3.92	3.82	2.64	2.27	2.11	2.02	2.75	3.06	2.72	2.90
Valine	3.70	3.92	2.79	2.41	2.63	3.51	3.31	3.51	3.22	2.90
Glycine	3.92	3.53	2.41	2.32	2.21	1.87	2.99	2.69	2.81	2.63
Isoleucine	3.02	3.39	2.15	2.02	1.78	2.17	2.89	2.78	2.63	2.48
Leucine	5.46	5.72	4.29	3.53	4.82	3.93	5.51	5.13	5.43	5.22
Proline	5.93	4.89	4.37	2.96	5.76	3.67	7.76	5.05	6.81	4.72
Threonine	2.95	2.94	1.84	1.76	2.31	1.83	2.60	2.50	2.56	2.51
Serine	4.17	3.26	2.81	2.15	3.75	2.31	4.25	2.89	4.02	3.07
Phenylalanine	2.92	3.17	2.30	2.00	2.24	2.16	2.50	2.87	2.65	2.91
Aspartic acid	6.42	6.58	5.77	4.71	4.18	4.00	5.20	5.09	5.33	5.48
Glutamic acid	17.02	13.00	9.52	8.62	12.70	9.92	14.22	13.65	14.31	13.55
Tyrosine	3.09	2.61	2.20	1.31	4.04	1.91	3.47	1.96	3.33	1.67
Lysine	4.49	4.65	3.52	2.78	3.55	2.96	5.12	4.32	4.83	4.00

[a] IE, ion-exchange chromatography; GLC, gas–liquid chromatography.

Table IX. Amino Acid Composition of 1-Day Mixed Food Diet Samples: Five Men (Values Are Expressed as g/Day)[a]

	D 18		D 19		D 20		D 21		D 22	
Amino acid	GLC	IE	GLC	IE	GLC	IE	GLC	IE	GLC	IE
Alanine	1.85	1.55	2.14	2.35	1.71	2.53	1.85	2.44	1.86	2.21
Valine	2.16	2.19	2.52	2.74	2.11	2.80	2.21	2.83	2.04	2.69
Glycine	1.65	1.73	2.22	2.27	1.78	2.18	2.00	2.10	1.78	1.90
Isoleucine	1.75	1.49	1.98	2.23	1.73	2.27	1.95	2.23	1.61	2.17
Leucine	3.30	3.10	4.25	3.94	3.34	3.88	4.17	4.17	3.35	3.78
Proline	3.57	2.76	4.81	3.38	4.10	3.58	4.19	3.17	3.46	4.01
Threonine	1.47	1.44	1.73	2.07	1.66	1.97	1.92	1.96	1.51	1.92
Serine	2.43	1.74	2.74	2.04	2.68	2.32	3.16	2.21	2.69	2.53
Phenylalanine	1.47	1.75	1.61	2.20	1.87	2.20	1.91	2.46	1.63	2.22
Aspartic acid	3.19	2.95	3.93	4.15	3.76	4.42	3.93	4.37	3.04	3.79
Glutamic acid	8.92	7.70	10.57	9.97	8.27	9.86	10.51	9.92	8.33	9.90
Tyrosine	1.72	1.11	1.70	1.56	2.36	1.64	2.04	1.33	1.75	1.46
Lysine	3.02	2.95	3.68	4.06	3.90	2.78	2.84	4.41	2.63	2.94

[a] IE, ion exchange chromatography; GLC, gas–liquid chromatography.

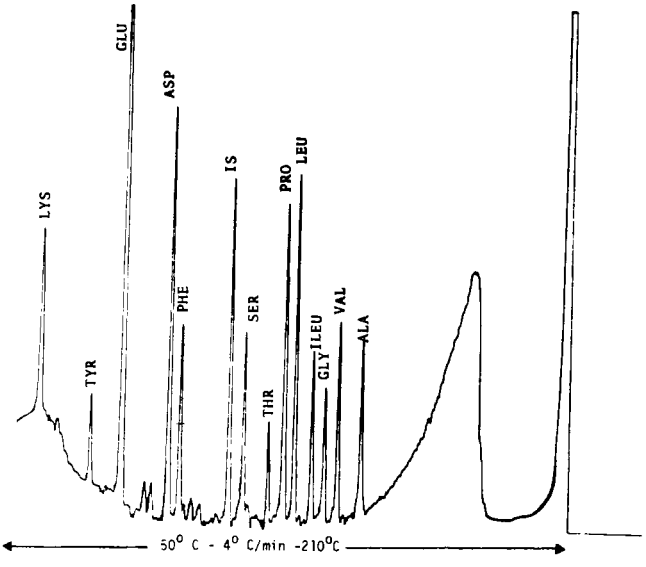

Figure 3. Separation of amino acids from a 1-day mixed food diet sample (13 g) as their TAB derivatives from an EGA column (for chromatographic conditions see Figure 1).

day during 1 week. Each day's protein was analyzed separately by gas chromatography (Figure 3) and the average intake per day was calculated. For analysis by ion-exchange chromatography, the daily portions were pooled together in proportion to their protein content and one subsample was drawn to represent a day's intake. The average values for amino acids by GLC show satisfactory agreement with the values obtained by analyzing pooled sample using ion-exchange chromatography. The intake of essential amino acids seems to be satisfactory in relation to the recommendations (FAO/WHO Ad Hoc Expert Committee, 1973). At present we are analyzing large numbers of duplicate portion diet samples for determining the daily intake of various amino acids by a population of pensioners of both sexes. The results are to be published later.

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Examination and Modification of the Use of *Leuconostoc mesenteroides* for Measurements of the Sulfur-Containing Amino Acids from *Vigna unguiculata*

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The use of *Leuconostoc mesenteroides* for the measurement of methionine and cyst(e)ine from the seed proteins of the legume *Vigna unguiculata* as well as *Phaseolus vulgaris* was examined and modified. Principal modifications include the use of buffers in neutralization and Pronase hydrolysis. Pronase appears useful in the hydrolysis of seed proteins of *V. unguiculata* but not for those from *P. vulgaris*. The method, as modified here, recovers as much methionine as that measured by an amino acid analyzer. It was found that hydrolysis preceding amino acid measurement need not be complete since the bacteria can obtain methionine from di- and tripeptides. Another advantage of this assay is that methionine derivatives, known to be nutritionally less desirable when fed to chicks, are shown to be less desirable when fed to this bacterium. Bacterial growth rates obtained on each of the derivatives mimic those reported for chicks. Because of differential growth given by equal amounts of cysteine and cystine, this method cannot give reliable measurements of these two amino acids. Further studies of this anomalous result suggest that cysteine must be converted to cystine before it supports bacterial growth. Other problems in the use of this assay are discussed.

When monogastric animals digest proteins from legumes, methionine appears to be the first limiting essential amino acid (Boulter et al., 1973). Cystine (cysteine) can apparently partially alleviate the requirement for methionine. Thus, any effort to improve the protein quality of legumes by genetic means must concentrate on the sulfur amino acids. Technically, a search for rare genotypes with elevated levels of these amino acids requires a rapid, reliable method for their measurement. The amino acid analyzer is not ideal since analysis is time consuming, exhaustive hydrolysis sometimes destroys methionine, and methionine derivatives known to be nutritionally less desirable in comparison to methionine are measured as methionine. An alternative method involves use of the bacterium *Leuconostoc mesenteroides*. This bacterium in a microbiological assay appears promising because a strain is available that requires both methionine and cystine (cysteine), and the appropriate growth media are available commercially. Moreover, this bacterium has been used for the measurement of these amino acids (Steel et al., 1949; Evans et al., 1974).

Although this bacterium has been used to measure the S-amino acids, we have found no literature pertaining to the accuracy and sensitivity of this method in measuring the total S-amino acid content. Thus, we have examined this microbiological assay, as well as hydrolysis preceding measurement, for the analysis of methionine and cystine from the legume, *Vigna unguiculata* (cowpea). Procedural modifications have been introduced to reduce the time required for amino acid measurement. The results of these studies, which are the subject of this report, strongly suggest that this method should be useful in programs where many samples are to be analyzed for methionine content.

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MATERIALS AND METHODS

The bacterium *Leuconostoc mesenteroides* ATCC 8042 was obtained from Difco Lab., Detroit, Mich., as were the methionine (Difco 423) and cysteine (Difco 467) growth media. Bacteria were routinely maintained in a 1:1 mixture of these media and in stab cultures of AOAC lactobacilli agar at 0–5 °C.

Materials for analysis were ground in a Udy mill, dried at 50 °C, and then stored in a desiccator. Hydrolysis of proteins was performed enzymatically or by means of 6 N HCl as described below.

Five milliliters of 6 N HCl was added to 100 mg of sample and the vessel was covered with a hard plastic Teflon-lined screw cap. Hydrolysis was carried out in an autoclave at 121 °C for 60 min. The samples were cooled, adjusted to pH 6.2 to 6.6 with 6 N NaOH, filtered with suction, and made up to volume (30–50 mL) with 0.2 M potassium–sodium phosphate (pH 6.4). The phosphate buffer does not affect bacterial growth.

For enzymatic hydrolysis, the enzyme solution was 0.01 M Tris-HCl, 0.15% Pronase (P-5130, Sigma), and 0.01 M $CaCl_2$ (pH 8.0). Five milliliters was added to 100 mg of sample and incubated at 37 °C for 1.5 h in a stationary water bath (continuous shaking does not increase the amount of amino acid recovery). After hydrolysis, the