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GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF PROTEIN AMINO ACIDS AS N-ISOBUTYLOXYCARBONYLAMINO ACID METHYL ESTERS

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SUMMARY

A practical method for the quantitative determination of protein amino acids by gas–liquid chromatography (GLC) is described. All of the common protein amino acids except arginine can be readily converted into their N-isobutyloxycarbonyl (N-isoBOC) methyl ester derivatives by a simple procedure involving isobutyloxycarbonylation with isobutyl chloroformate in aqueous medium, followed by methylation with diazomethane. Arginine was converted into N-isoBOC ornithine methyl ester by treatment with arginase, followed by the above derivatization procedure. The resulting N-isoBOC methyl esters of the amino acids have good GLC properties. Complete resolution of the derivatives of 20 protein amino acids was achieved by using a dual-column system consisting of a 0.65% Poly-A-101A column and a 0.70% FFAP–Poly-A-101A (1:1, w/w) column. The reproducibility of response was found to be good for derivatives carried through the entire chemical and chromatographic procedure. The calibration graphs were linear and showed no statistical bias. The results of recovery experiments with synthetic mixtures containing known amounts of the amino acids were satisfactory, the recoveries ranging from 94.3 to 106.2%.

INTRODUCTION

The application of gas–liquid chromatography (GLC) to the analysis of amino acids has been the subject of extensive investigations[1–3] because of its great potential in providing a means by which qualitative and quantitative analyses of amino acids can be effected rapidly and accurately in comparison with other techniques.

However, the development of a practical GLC method has been hindered by the difficulty of converting a variety of amino acids in quantitative yield into volatile derivatives suitable for GLC by general and convenient techniques. Although several refined GLC methods for analyzing quantitatively all of the common protein amino acids have been reported[4–11], the usefulness of these methods for the routine analysis of amino acids has not yet obtained general acceptance.

We have now developed a GLC method for the routine analysis of amino acids, based on the preparation and chromatography of the N-isobutyloxycarbonyl (N-isoBOC) methyl ester derivatives. As the preparation of the N-isoBOC methyl

esters of the amino acids is rapid and convenient, the complete analysis of protein amino acids by GLC has become a practical laboratory procedure. A preliminary account of this investigation has appeared elsewhere[12].

EXPERIMENTAL

Reagents

All of the amino acids used in this study, except isoleucine and hydroxyproline, were purchased from Nakarai Chemicals (Kyoto, Japan). Isoleucine and hydroxyproline were purchased from Wako (Osaka, Japan). Ethyl, isobutyl, *n*-butyl, *n*-amyl, *n*-hexyl and *n*-octyl chloroformates stabilized with calcium carbonate were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and used without further purification. N-Methyl-N-nitroso-*p*-toluenesulphonamide for the evolution of diazomethane was purchased from Wako.

Diethyl ether. Commercial diethyl ether (1 l) was treated three times with 50 ml of acidic iron(II) sulphate solution [($FeSO_4 \cdot 7H_2O$ (120 g) + 95% H_2SO_4 (12 ml) + H_2O (220 ml)] in order to remove peroxides, and the treated diethyl ether was distilled in an all-glass apparatus after being washed with water and dried over anhydrous sodium sulphate. The peroxide-free diethyl ether thus obtained was stored in an amber-glass bottle at 4°.

Arginase. Arginase (40–60 units/mg, from bovine liver) was purchased from Sigma (St. Louis, Mo., U.S.A.). Before use, 5 mg of enzyme was activated by treatment with 4.5 ml of 0.1 *M* ammonium acetate and 0.5 ml of 0.5 *M* manganese(II) sulphate at 37° for 4 h. The solution of activated enzyme could be stored frozen without loss of activity for at least 15 days.

Standards. Two standard solutions, one containing 20 amino acids and another containing the 20 amino acids plus ornithine, were prepared so as to contain 0.5 mg/ml of each amino acid in 0.5% hydrochloric acid. Working solutions were prepared by diluting these solutions with distilled water so as to give a concentration of 100 μg/ml of each amino acid.

The internal standard, norleucine (Nakarai Chemicals), was used as the most convenient pure internal standard available. A working standard solution in 0.1% hydrochloric acid was prepared at a concentration of 100 μg/ml.

All standard solutions were stored in capped glass bottles maintained at 4°.

Columns

The liquid phases Poly-A-101A, OV-1 and OV-17 and the support, 100–120 mesh Gas-Chrom P, were purchased from Applied Science Labs. (State College, Pa., U.S.A.), and the liquid phase FFAP from Varian (Instrument Division, Palo Alto, Calif., U.S.A.). The Gas-Chrom P, glass columns and quartz-wool plugs placed in each end of the column used throughout this work were silanized with 5% of dimethyldichlorosilane in toluene. The column packings, 0.70% FFAP–Poly-A-101A (1:1, w/w) on Gas-Chrom P and 0.65% Poly-A-101A on Gas-Chrom P, were prepared using *n*-butanol–chloroform (1:1) as a coating solvent according to the procedure of Horning *et al.*[13]. The former was packed in a 2 m × 3 mm I.D. glass column and the latter in a 1 m × 3 mm I.D. glass column. They were pre-conditioned with a nitrogen flow of *ca.* 20 ml/min at 275° for 24 h. All other column packings used in this work

TABLE I

GLC OPERATING CONDITIONS

Condition	*Column*	
	0.65% Poly-A-101A	*0.70% FFAP–Poly-A-101A (1:1, w/w)*
Initial temperature (°C)	80	100
Isothermal time (min)	5	5
Temperature programming rate (°C/min)	4	4
Final temperature (°C)	270	260*
Injection and detector temperature (°C)	270	270
Carrier gas (N_2) flow-rate (ml/min)	40	40
Hydrogen flow-rate (ml/min)	50	50
Air flow-rate (ml/min)	750	750
Attenuation	$10^2 \times 32$	$10^2 \times 32$

* Soon after elution of norleucine, the column temperature was increased quickly to 260° by manual operation.

were prepared and pre-conditioned at an appropriate temperature for each liquid phase in the same way.

Chromatography

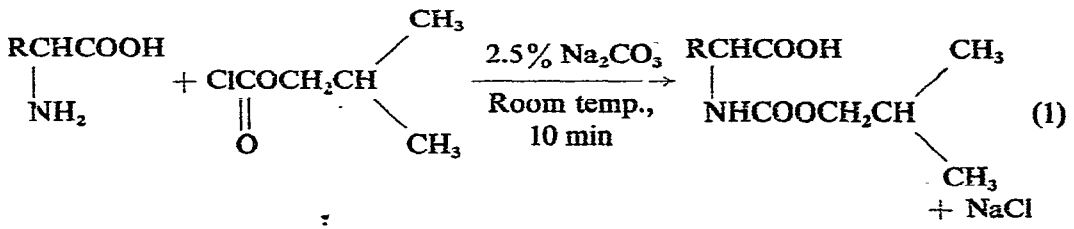
A Shimadzu 5A gas chromatograph equipped with a dual-column oven bath, a dual differential hydrogen flame detector, on-column injection ports and a linear temperature programmer (Shimadzu TP-5) was used. The operating conditions are given in Table I. Two columns as described above were connected to the gas chromatograph as a pair and operated in either an analytical or a reference mode.

Mass spectrometry

The mass spectra of the N-isoBOC methyl ester derivatives of the protein amino acids were measured using a Shimadzu LKB 9000 gas chromatograph–mass spectrometer with 2 m × 3 mm I.D. glass column packed with 1% OV-1 or 3% OV-17 on acid-washed and silanized 80–100 mesh Chromosorb W (Johns-Manville, Denver, Colo., U.S.A.). Each derivative was chromatographed isothermally using either an OV-1 or OV-17 column at 180–240°, depending on the volatility differences. The operating conditions were follows: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 270°; separator temperature, 250°.

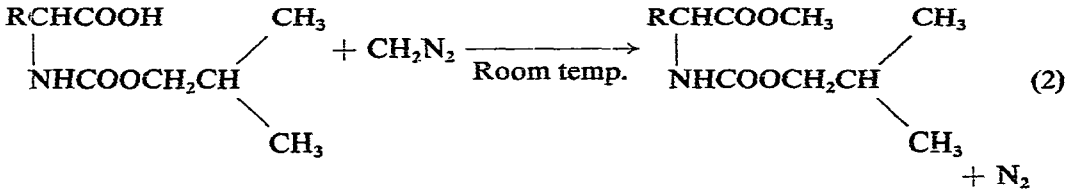
Analytical derivatization method

An aqueous aliquot containing up to *ca.* 2 mg of total amino acids (from 10 to 100 μg of each amino acid) and 0.5 ml of the internal standard solution were pipetted into a 10-ml polyethylene-stoppered vial. To this solution, 0.5 ml of 10% sodium carbonate solution was added and the total volume was made up to 2 ml with distilled water. Then 0.1 ml of isobutyl chloroformate (isoBCF) was added immediately and the reaction mixture was shaken with a shaker for 10 min at room temperature, when the following reaction occurred:



(1)

The product was extracted twice with 3 ml of diethyl ether in order to remove the excess of reagent, and the ethereal extracts were discarded. The aqueous layer was saturated with sodium chloride, acidified to pH 1–2 with 10% orthophosphoric acid (thymol blue test paper) and then extracted three times with 3 ml of diethyl ether with vigorous shaking by hand for 1 min. To the combined ethereal extracts was added 1 ml of methanol, and methylation was carried out by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman[14], through this solution until a yellow tinge became visible according to the reaction:



(2)

After standing at room temperature for 5 min, the solvents were quickly evaporated to dryness at 50° under a stream of nitrogen. The residue was dissolved in an appropriate amount of ethyl acetate (0.1–0.2 ml), the solution was dried over anhydrous sodium sulphate and 4 μl of the solution was injected on to the gas chromatograph.

Conversion of arginine into ornithine

An aqueous aliquot containing up to 100 μg of arginine and 0.5 ml of the internal standard solution were pipetted into a polyethylene-stoppered vial and the total volume was made up to *ca.* 1.5 ml with distilled water. The solution was adjusted to pH 9.4–9.6 with 5% sodium carbonate solution (thymol blue test paper) and to this solution was added 0.1 ml of the solution of activated arginase. The mixture was incubated for 20 min at 37° with occasional shaking, and then subjected to the analytical derivatization method.

Synthesis of reference compounds

Pure N-isoBOC methyl ester derivatives of valine, threonine, aspartic acid, phenylalanine, cysteine, ornithine, lysine, tyrosine and tryptophan were synthesized for use as reference standards. One gram of the amino acid, 200 ml of 2.5% sodium carbonate solution and 5 ml of isoBCF were successively placed in a 500-ml separating funnel and the mixture was shaken with a shaker for 15 min at room temperature. The reaction mixture was extracted twice with 50 ml of diethyl ether and the ethereal extracts were discarded. The aqueous layer was acidified (pH *ca.* 2) with 10% orthophosphoric acid solution and extracted three times with 50 ml of diethyl ether. The combined ethereal extracts were dried over anhydrous sodium sulphate and concen-

trated to *ca.* 30 ml at 50° in a water-bath. After the addition of a one-tenth volume of methanol to the solution, methylation was carried out with diazomethane and then the solvents were evaporated to dryness at 50° in a water-bath. The residue was purified by vacuum distillation or recrystallization.

RESULTS AND DISCUSSION

Preliminary experiments on selection of derivative

A series of N-alkyloxycarbonyl [ethyloxycarbonyl (EOC), isoBOC, *n*-butyloxycarbonyl (*n*-BOC), *n*-amyloxycarbonyl (*n*-AOC), *n*-hexyloxycarbonyl (*n*-HOC) and *n*-octyloxycarbonyl (*n*-OOC)] methyl ester derivatives of the protein amino acids were prepared according to the derivatization method described above and their GLC properties were evaluated.

As expected, the N-EOC derivative was found to be the most volatile derivative, but difficulty was encountered in its quantitative formation under the conditions of the derivatization method. The N-isoBOC, N-*n*-BOC, N-*n*-AOC derivatives were found generally to be satisfactory in terms of both conversion yield and GLC properties. However, with N-*n*-BOC and N-*n*-AOC derivatives, a linear calibration graph for tyrosine could not be obtained. The N-*n*-HOC and N-*n*-OOC derivatives were found to be insufficiently volatile for use in the complete analysis of the protein amino acids. On the basis of these results, the N-isoBOC derivative was selected for subsequent study.

Derivatization reaction

In order to determine the optimum derivatization reaction conditions, the effects of the concentration of the reagents and the reaction time were studied. It was found that 0.1 ml of isoBCF was sufficient for quantitative isobutyloxycarbonylation of at least up to 20 mg of a mixture of amino acids. Sodium carbonate was chosen as the alkaline reagent for the isobutyloxycarbonylation and its optimum concentration was found to lie in the range 2–3%. The use of sodium hydroxide was not preferred because instability of isoBCF in reaction mixtures containing sodium hydroxide was observed. The relative weight response (RWR) of the amino acids as a function of isobutyloxycarbonylation time was studied and, based on the result, a reaction time of 10 min was selected. The use of diethyl ether as extraction solvent is advantageous because subsequent esterification is carried out in the same solvent and removal of the solvent can be effected quickly at a relatively low temperature. However, diethyl ether that contained peroxides caused partial oxidation of the methionine derivative to its sulphoxide, and therefore the use of peroxide-free diethyl ether was essential for successful analysis. It should be noted that the extraction of reaction mixtures with diethyl ether at alkaline pH (prior to acidification with orthophosphoric acid) serves to remove the isoBOC derivatives of amines and phenols (not containing a carboxylic or sulphonic acid function in the molecule) and also excess of isoBCF, and therefore amino acids can be analyzed by the method described without interference from amines and phenols*, both of which often coexist with amino acids in biological samples.

* The GLC of amines and phenols as their isoBOC derivatives will be reported in subsequent papers.

TABLE II

ELEMENTAL ANALYSES OF N-isoBOC METHYL ESTERS OF AMINO ACIDS

Amino acid	*Empirical formula*	*Molecular weight*	*Analysis (%)*						*B.p. (°C/mmHg)*	*M.p. (°C)*
			C		*H*		*N*			
			Calculated	*Found*	*Calculated*	*Found*	*Calculated*	*Found*		
DL-Val	$C_{11}H_{21}O_4N$	231.29	57.12	56.83	9.15	9.13	6.06	6.00	95.0–96.0/2	
L-Asp	$C_{11}H_{19}O_6N$	261.27*	50.56	50.29	7.33	7.33	5.36	5.34	145.5–147.0/3	
L-Thr	$C_{10}H_{19}O_5N$	233.26	51.49	51.74	8.21	8.17	6.01	6.08	135.0–136.5/2	47.2–48.0***
DL-Phe	$C_{15}H_{21}O_4N$	279.33	64.49	64.30	7.58	7.51	5.01	5.13	137.5–138.0/2	
L-Cys	$C_{14}H_{25}O_6NS$	335.41**	50.12	49.87	7.51	7.47	4.17	4.21	159.0–160.0/2	
L-Orn	$C_{16}H_{30}O_6N_2$	346.42**	55.47	55.15	8.73	8.66	8.09	8.18	185.0–186.5/2	
L-Lys	$C_{17}H_{32}O_6N_2$	360.43**	56.64	56.19	8.95	8.95	7.77	7.48	195.0–196.0/2	
L-Tyr	$C_{20}H_{29}O_7N$	395.44**	60.74	60.99	7.39	7.42	3.54	3.63		53.0–54.0‡
L-Trp	$C_{17}H_{22}O_4N_2$	318.36	64.13	64.43	6.97	6.96	8.80	8.81		79.5–80.5‡

* Calculated for the dimethyl ester.

** Calculated for the diisoBOC ester.

*** Needles, recrystallized from *n*-hexane–diethyl ether.

‡ Needles, recrystallized from *n*-hexane–acetone.

TABLE III

PERCENTAGE CONVERSIONS OF AMINO ACIDS INTO THEIR N-isoBOC METHYL ESTERS

Amino acid	*Yield of derivatives (%)**					
	*Individual***			*Mixture****		
	1§	*2*§	*Average*	*1*§	*2*§	*Average*
Val	99.4	99.8	99.6	98.4	102.4	100.4
Asp	104.8	97.7	101.3	97.8	99.6	98.7
Thr	95.5	97.9	96.7	98.6	97.3	98.0
Phe	101.7	103.4	102.6	99.2	98.3	98.8
Cys	92.8	97.6	95.2	95.8	98.9	97.4
Orn	96.6	97.3	97.0	95.5	99.5	97.5
Lys	97.9	98.1	98.0	102.4	103.0	102.7
Tyr	95.6	94.1	94.9	95.1	96.9	96.0
Trp	99.3	100.5	99.9	103.1	101.2	102.2

* The yield was calculated by comparing the peak height obtained for the derivative prepared by the analytical procedure with that obtained for the pure derivative.

** Amino acids were derivatized individually.

*** Amino acids were derivatized in the presence of equal amounts of each of the other protein amino acids.

§ 1 and 2 are independent samples.

In order to assess the yield of derivatives in the derivatization method, pure reference derivatives of nine representative amino acids were synthesized. The results of elemental analyses of the amino acid derivatives are shown in Table II. The retention time of each reference derivative was in fair agreement with that of the corresponding analytical derivative. Percentage conversions of the nine amino acids into their N-isoBOC methyl ester derivatives are given in Table III. Good results were obtained, the yield being above 92% in each instance. The fact that the yields obtained when each amino acid is derivatized individually are essentially the same as those obtained when each amino acid is derivatized together with all other amino acids indicates that there are no problems due to mutual interferences in the analysis of mixtures.

Conversion of arginine into ornithine

It was impossible to obtain the volatile derivative of arginine by direct use of the derivatization method described above, but this problem was overcome by converting arginine into ornithine with arginase prior to derivatization. The procedure reported here for the conversion is essentially the same as that of Coulter and Hann[15]. However, in view of the convenience of the overall process, the method described here is superior to the Coulter and Hann method, in which the N-acetyl *n*-propyl ester derivative is utilized, as their method requires complete exclusion of water prior to subsequent derivatization, while our method does not require such a troublesome process.

Percentage conversions of arginine into ornithine are shown in Table IV. The yield was above 93% at each level of arginine from 10 to 150 μg, and the presence of other protein amino acids did not inhibit the enzymatic reaction.

TABLE IV

PERCENTAGE CONVERSIONS OF ARGININE INTO ORNITHINE BY ARGINASE TREATMENT

*Amount of arginine treated** (μg)*	*Conversion yield (%)***				
	*1****	*2****	*3****	*4****	*Average*
10	94.5	95.3	99.3	99.2	97.1
25	99.0	98.6	98.0	96.4	98.0
50	97.7	97.9	100.7	101.6	99.5
100	97 4	102.5	97.4	99.2	99.1
150	93.4	94.1	98.4	97.9	96.0

* The arginine standard solution used in this experiment contained equal amounts of all other protein amino acids to arginine.

** Amounts of ornithine formed were determined by GLC and the yields were calculated from the values.

*** 1, 2, 3 and 4 are independent samples.

Separation of N-isoBOC methyl ester derivatives

The chromatographic and instrumental conditions for the resolution of the N-isoBOC methyl esters of the common protein amino acids were investigated in detail. Among the liquid phases investigated were OV-1, OV-17, OV-25, neopentyl glycol succinate, cyclohexane dimethanol succinate, Versamide 930, FFAP, Poly-A-101A, Poly-A-103 and Poly-I-110. There was neither a single liquid phase nor a single column that resolved all 20 amino acid derivatives. However, complete separation and analysis could be achieved by using a dual-column system consisting of a 0.65% Poly-A-101A column and a 0.70% FFAP–Poly-A-101A (1:1, w/w) column. The 0.65% Poly-A-101A column separated 16 of the amino acids (all except glycine, valine, isoleucine and proline). On the other hand, glycine, valine, isoleucine, proline and a number of others could be resolved on the 0.70% FFAP–Poly-A-101A column.

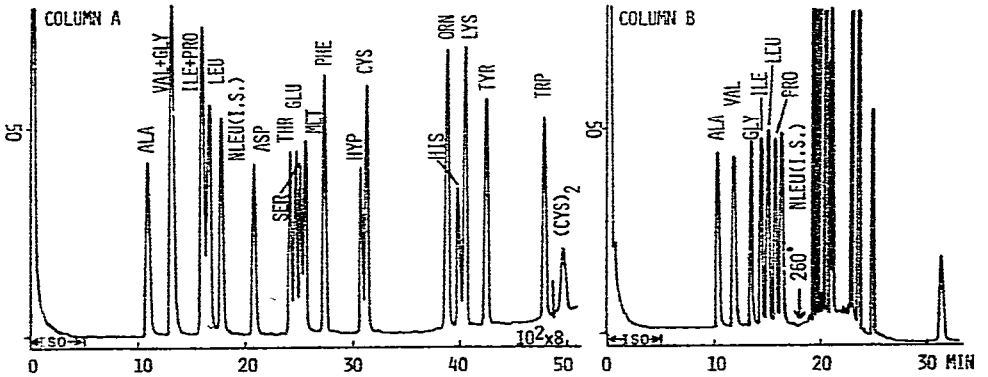


Fig. 1. Separation of the N-isoBOC methyl esters of the 20 protein amino acids with a dual-column system. Column A: 0.65% Poly-A-101A on 100–120 mesh silanized Gas-Chrom P, 2 m × 3 mm I.D., glass. Column B: 0.70% FFAP–Poly-A-101A (1:1, w/w) on 100–120 mesh silanized Gas-Chrom P, 1 m × 3 mm I.D., glass. Conditions: see Table I. Each peak represents *ca.* 2 μg of amino acid.

The internal standard, norleucine, was completely separated from the 20 amino acids on both of these columns. In practice, the 0.70% FFAP–Poly-A-101A column was used for the analysis of alanine, valine, glycine, isoleucine, leucine and proline, and the 0.65% Poly-A-101A column was used for the analysis of the remaining 14 amino acids. A chromatogram of the complete separation of the 20 protein amino acids with a dual column system is shown in Fig. 1. The peak of the cystine derivative obtained with the Poly-A-101A column was relatively small compared with those of the other amino acid derivatives, but this problem was satisfactorily compensated for by increasing the sensitivity of the instrument just before the elution of this derivative. The low response of the cystine derivative is considered to be due to interaction of the derivative with the polar liquid phase, Poly-A-101A, as the response was greatly improved when a non-polar phase such as OV-1 was used.

Stability of N-isoBOC methyl ester derivatives

The stability of the N-isoBOC methyl esters of the amino acids with respect to time was found to be good. The recovery, as determined by peak height, was essentially quantitative for all 20 of the protein amino acid derivatives, which had been allowed to stand in solution in ethyl acetate for 1 week at room temperature. It should be emphasized that all of the derivatives are very stable towards moisture and therefore no precautions to exclude moisture are necessary in their handling and storage.

TABLE V

RELATIVE WEIGHT RESPONSES (RWR) OF N-isoBOC METHYL ESTERS OF AMINO ACIDS*

Amino acid	*RWR***					*RSD (%)*
	*1****	*2****	*3****	*4****	*Average*	
Ala	0.871	0.861	0.862	0.871	0.866	0.64
Val	0.884	0.882	0.896	0.874	0.884	1.03
Gly	0.962	0.959	0.949	0.967	0.959	0.79
Ile	0.989	0.979	0.967	0.977	0.978	0.92
Leu	1.052	1.051	1.064	1.065	1.058	0.71
Pro	0.952	0.955	0.963	0.954	0.956	0.51
Asp	0.778	0.781	0.784	0.794	0.784	0.89
Thr	0 871	0.879	0.889	0.873	0.878	0.92
Glu	0.848	0.859	0.862	0.864	0.858	0.83
Ser	0.744	0.738	0.730	0.739	0.738	0.79
Met	0.862	0.884	0.854	0.857	0.864	1.57
Phe	1.184	1.183	1.170	1.190	1.182	0.71
Hyp	0.751	0.756	0.760	0.741	0.752	1.09
Cys	1.188	1.180	1.204	1.189	1.190	0.84
Orn	1.393	1 372	1.362	1.357	1.371	1.15
His	0.693	0.667	0.671	0.653	0.671	2.47
Lys	1.393	1.419	1.416	1.429	1.414	1.08
Tyr	1.084	1.087	1.061	1.075	1.077	1.08
Trp	0.973	0.951	0.970	0.963	0.964	1.01
$(Cys)_2$	0.319	0.329	0.324	0.360	0.333	5.54
Arg	1.033	1.019	1.042	1.046	1.035	1.16

* Relative to norleucine derivative.

** RWR = (peak height amino acid/weight amino acid)/(peak height nleu/weight nleu).

*** 1, 2, 3 and 4 are independent samples.

TABLE VI

CALIBRATION LINEARITY FOR AMINO ACIDS AS N-isoBOC METHYL ESTERS

Amino acid	*Amount derivatized (μg)*							
	10		*25*		*50*		*100*	
	*(RWR) (%)**	*RSD (%)*	*(RWR) (%)**	*RSD (%)*	*(RWR) (%)**	*RSD (%)*	*(RWR) (%)**	*RSD (%)*
Ala	9.4	1.56	23.9	1.57	50.2	0.63	100	0.33
Val	9.6	0.84	24.1	1.24	50.5	0.99	100	0.82
Gly	9.2	1.01	24.4	0.97	50.9	0.83	100	1.12
Ile	9.7	1.79	25.4	0.38	51.2	0.88	100	0.93
Leu	10.2	0.49	24.9	0.87	50.4	0.75	100	0.52
Pro	10.1	1.40	25.0	0.95	49.9	0.54	100	0.63
Asp	10.4	0.92	25.1	1.32	49.9	0.87	100	0.89
Thr	9.5	0.48	25.8	1.11	51.7	0.97	100	0.14
Glu	10.5	1.80	25.8	0.45	50.8	0.79	100	0.92
Ser	9.3	0.48	25.9	1.39	49.4	0.93	100	0.90
Met	10.0	1.91	25.2	0.86	49.3	1.56	100	0.43
Phe	10.0	1.00	25.8	1.02	50.1	0.68	100	0.54
Hyp	9.6	2.33	25.8	0.80	50.1	1.11	100	0.94
Cys	10.7	2.35	25.1	1.17	51.7	0.80	100	0.80
Orn	10.9	0.75	25.2	0.94	50.3	1.14	100	0.82
His	9.6	2.48	25.7	1.46	50.5	2.45	100	2.23
Lys	10.1	0.81	25.4	0.50	53.1	1.05	100	0.55
Tyr	10.9	2.74	23.6	1.09	51.2	1.12	100	1.00
Trp	10.4	1.76	25.3	0.81	51.0	1.03	100	0.22
$(Cys)_2$	11.1	6.51	27.0	5.19	54.3	5.47	100	1.17
Arg	10.5	1.23	25.4	0.96	50.7	0.98	100	1.02
Theory	10.0		25.0		50.0		100	

* (RWR) (%) = [RWR value at each level/RWR value at highest level (at 100 μg)]·100. Each value represents an average of four independent determinations.

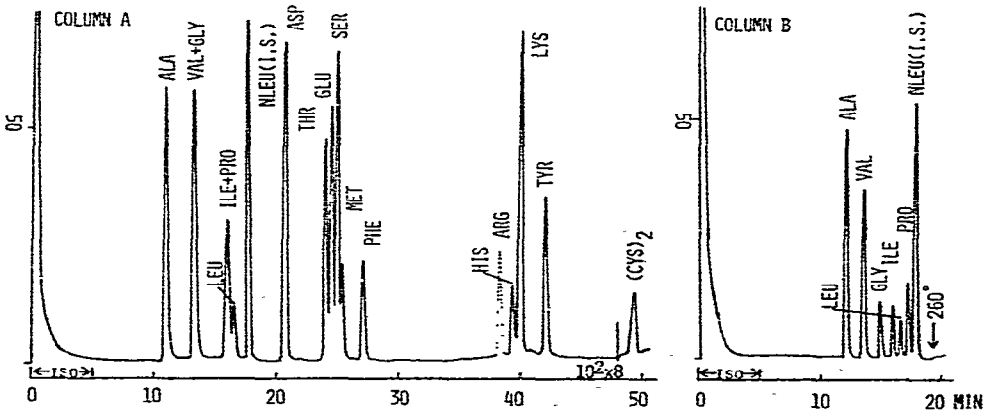


Fig. 2. Chromatogram obtained from a synthetic mixture of amino acids Sample: synthetic mixture A as in Table VII, *ca.* 40 μg of total amino acids injected. For further information, see legend to Fig. 1.

Structures of N-isoBOC methyl ester derivatives

The structures of the N-isoBOC methyl esters of the protein amino acids prepared by the analytical procedure were elucidated by gas chromatography–mass spectrometry. A molecular ion peak (M^+) was observed for each of the derivatives and other common ion peaks which were useful for structure elucidation were $M^+ - 15$ (CH_3), $M^+ - 59$ ($COOCH_3$) and $M^+ - 101$ [$COOCH_2CH(-CH_3)_2$]. Further, for some amino acids, the structure of the derivatives was confirmed by elemental analyses (see Table II). The results can be summarized as follows: (1) all carboxyl groups are esterified; (2) all amino, imino, imidazole, phenolic hydroxyl and sulphydryl groups are isobutyloxycarbonylated; and (3) alcoholic hydroxyl groups and indole ring nitrogen atoms are not isobutyloxycarbonylated.

Quantitation studies

Table V gives the RWR values relative to the norleucine derivative. Four replicate samples at the 50-μg level were derivatized and analyzed. The relative standard deviation (RSD) was less than 2.5% for all protein amino acids except cystine, for which the value was 5.54%.

In order to test the linearity of the calibration graph, four replicate samples at each level in the range 10–100 μg were derivatized and analyzed. As shown in Table VI, the linearity of the calibration graph for each amino acid in the range studied and its reproducibility were found to be satisfactory.

TABLE VII

RECOVERY OF AMINO ACIDS FROM SYNTHETIC MIXTURES CONTAINING KNOWN AMOUNTS OF AMINO ACIDS

Amino acid	*A*			*B*			*C*		
	Added (mg)	*Found* (mg)*	*Recovery (%)*	*Added (mg)*	*Found* (mg)*	*Recovery (%)*	*Added (mg)*	*Found* (mg)*	*Recovery (%)*
Ala	9.7	10.13	104.4	7.6	7.48	98.4	9.0	9.29	103.2
Val	7.3	7.72	105.8	7.0	7.03	100.4	9.4	9.60	102.1
Gly	2.4	2.33	97.1	1.8	1.91	106.1	9.4	9.84	104.7
Ile	2.4	2.31	96.3	2.1	1.98	94.3	4.1	4.02	98.0
Leu	1.6	1.59	99.4	2.2	2.23	101.4	7.8	7.96	102.1
Pro	3.2	3.21	100.3	3.1	3.06	98.7	3.7	3.75	101.4
Asp	12.1	12.45	102.9	14.1	13.71	97.2	9.4	9.47	100.7
Thr	8.1	8.33	102.8	7.8	8.00	102.6	9.4	9.70	103.2
Glu	9.7	10.04	103.5	12.1	12.25	101.2	6.1	6.48	106.2
Ser	12.1	12.05	99.6	9.4	9.93	105.6	11.9	12.01	100.9
Met	3.2	3.31	103.4	3.4	3.25	95.6	0.8	0.77	96.3
Phe	2.4	2.47	102.9	3.6	3.55	98.6	2.5	2.45	98.0
Arg	3.2	3.19	99.7	4.7	4.54	96.6	1 6	1.64	102.5
His	3.2	3.12	97.5	3.5	3.30	94.3	0.8	0.78	97.5
Lys	8.1	8.46	104.4	10.6	10.96	103.4	5.7	5.82	102.1
Try	4.8	4.67	97.3	6.8	6.50	95.6	1.6	1.51	94.4
Trp	—**			—**			3.3	3.47	105.2
(Cys)$_2$	6.5	6.14	94.5	6.2	6.47	104.4	4.1	4.19	102.2

* Each value represents the average of two independent determinations.

** Not added.

The quantification of the amino acids by the method described was established from the analysis of three synthetic mixtures containing known amounts of amino acids. A chromatogram obtained from a synthetic mixture is shown in Fig. 2. The percentage recovery of each amino acid, as calculated by comparison of the values obtained from the analysis with theoretical values, is given in Table VII. The recovery was reasonable, ranging from 94.3 to 106.2%.

The application of the method described to the determination of amino acids in biological substances will be reported in subsequent papers.

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