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Short Communication

Determination of 22 protein amino acids as N(O)-*tert.*-butyldimethylsilyl derivatives by gas chromatography

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ABSTRACT

Twenty-two protein amino acids were simultaneously derivatized to N(O)-*tert.*-butyldimethylsilyl derivatives by a single-step reaction at 75°C for 30 min with the silylating reagent N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide. All of the derivatives were quantitatively resolved in 41 min by GC on DB-1 capillary column. The relative standard deviations of the relative molar response with regard to the internal standard (pyrene) were less than 5% for all of the derivatives. Calibration graphs showed good linearity in the measured range. Correlation coefficients and regression coefficients for all of the calibration graphs were highly significant ($p < 0.001$). The mass spectra of the derivatives showed that all of the amino acids were derivatized to the N(O)-*tert.*-butyldimethylsilylamino acids.

INTRODUCTION

The advantages of a gas chromatographic (GC) analysis of amino acids are obviously the low cost, the high sensitivity and the much greater versatility of the instrument compared with specialized amino acids analysers [1]. Moreover, GC offers a relatively simple means of combining the analytical system with mass spectrometry [2]. A disadvantage, however, is the necessity to derivatize the amino acids into more volatile, less polar compounds that are suitable for GC separation. Therefore, not only the development of GC systems but also the concomitant development of suitable derivative reactions are factors that lead to successful separations.

In most of the recent work in this field, the derivatization was carried out by more than two-step reactions, based on acylation of the α-amino group and esterification of the carboxyl group. Even though the GC analysis of acylated amino acid alkyl esters has become routine, there are some negative aspects of the derivatization procedures such as two incompatible reaction media with an intermediate evapora-

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tion step, degradation of the amides, glutamine and asparagine, due to the HCl catalyst, low solubilities of some amino acids in the higher alcohols and high reaction temperatures [3]. Therefore, the quantitative derivatization of all the functional groups of protein amino acids by one-step reaction has been studied. A relatively successful method was the trimethylsilylation (TMS) procedure with the silylating agent bis-(trimethylsilyl)trifluoroacetamide (BSTFA) [4]. However, two different reaction times were required to derivatize quantitatively the protein amino acids. Fourteen amino acids could be silylated in 15 min at 135°C, whereas six amino acids (glutamic acid, arginine, lysine, histidine, tryptophan and cysteine) required 4 h at the same temperature. Glutamine and aspargine were also not detected as TMS derivatives. Glutamine, asparagine and their acids have been successfully derivatized to N(O)-*tert.*-butyldimethylsilyl (tBDMSi) derivatives by N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and quantitatively separated on an SPB-1 fused-silica capillary column by GC [5]. Since then, the analysis of tBDMSi amino acid derivatives by capillary GC have been successfully accomplished for standard protein amino acids [6–9], protein hydrolysates and physiological samples [6,10–13]. Mass spectra of the derivatives were also obtained in several studies [7,8,10,11]. However, we were not able to find a successful report of quantitative analysis and a single-step derivatization method under mild conditions for all 22 protein amino acids. Recently, the GC analysis of N(O,S)-ethoxycarbonyl amino acid ethyl ester derivatives obtained with ethyl chloroformate was accomplished for 21 protein amino acids, but derivatization of arginine was incomplete and it failed to elute from the column [14,15].

The objective of this investigation was to study the derivatization of the 22 protein amino acids simultaneously in one reaction step with MTBSTFA and the quantitative separation of the derivatives on a DB-1 capillary column by GC. To identify the tBDMSi amino acid derivatives mass spectra of the derivatives also were studied.

EXPERIMENTAL

Materials

N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Aldrich (Milwaukee, WI, USA). Standard amino acids were obtained from Sigma (St. Louis, MO, USA). All other reagents and solvents were of analytical-reagent grade.

Derivatization

A mixture of standard amino acids (10 μl of solution containing 2.5 μmol/ml) was placed in a 1-ml conical vial and the solvent was completely evaporated at 50°C with dry nitrogen. Exactly 25 μl of internal standard (I.S.) solution (2.5 μmol of pyrene/ml of pyridine), 15 μl of MTBSTFA and 2 μl of triethylamine were added in that order. After tightly capping the vial, it was heated at 75°C for 30 min. After cooling to room temperature, the reaction mixture was injected directly on to the GC column.

Gas chromatography

All chromatography was accomplished with a Hitachi 163 gas chromatograph equipped with a capillary injection system with a split injector and a flame ionization detector. A DB-1 (100% methylsiloxane) fused-silica capillary column (60 m × 0.35 m I.D., 0.25 μm film thickness) (J & W Scientific) was used. The carrier gas (hydrogen) flow-rate was 2 ml/min. The splitting ratio was 5:1. The make up gas was nitrogen at a flow-rate of 30 ml/min.

Mass spectrometry

Mass spectra of tBDMSi amino acid derivatives were obtained using an HP 5971 mass-selective detector operated in the electron impact (EI) ionization mode. The ionization energy was 70 eV. A DB-1 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used. The carrier gas (helium) flow-rate was 0.9 ml/min. The other conditions were same as for GC.

RESULTS AND DISCUSSION

Chromatography

The chromatographic elution of the derivatives of a mixture of standard amino acids is shown in Fig. 1. All of the 22 protein amino acids were resolved in 41 min. Especially glutamine and asparagine were completely separated from their acids. The results for these four derivatives was similar to those reported previously [6,9–11]. Incompletely resolved derivatives were isoleucine and threonine, hydroxyproline-1 and cysteine and tryptophan and tyrosine. The hydroxyproline derivative showed multiple peaks. The peak of the cystine derivative appeared at about 300°C; this derivative seems less volatile than the others.

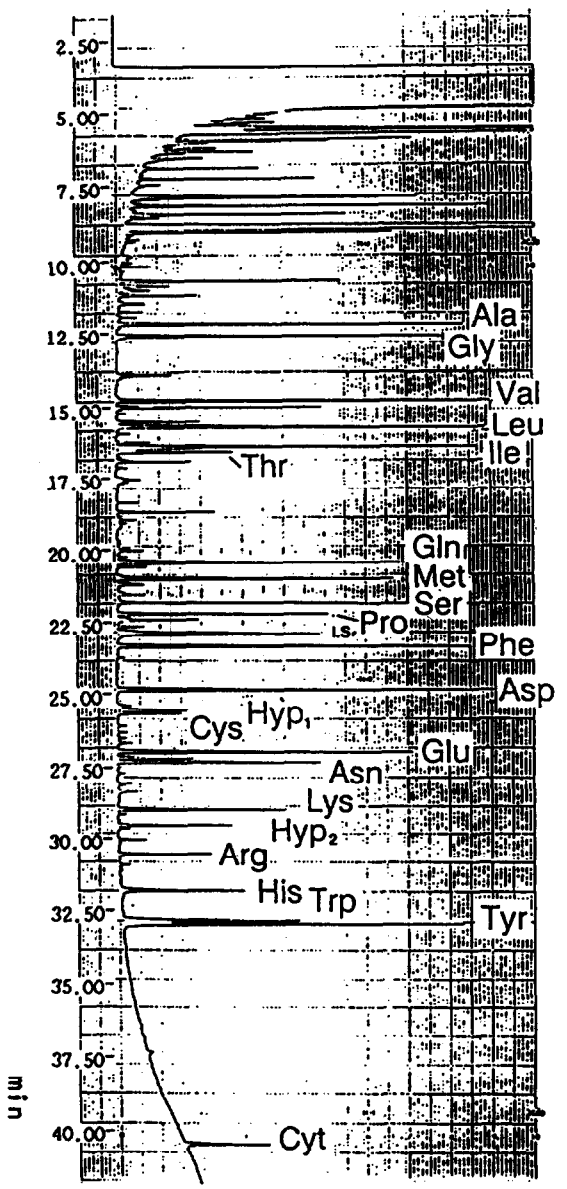

Fig. 1. Gas chromatogram of N(O)-*tert.*-butyldimethylsilyl-amino acids obtained on a DB-1 capillary column. Amount injected, 600 pmol, except cystine (1.2 nmol) and I.S. (750 pmol).

Relative molar response (RMR) and calibration graphs

The molar responses relative to pyrene are given in Table I. Four replicate samples were analysed. The amount of each amino acid injected was 600 pmol and that of pyrene (I.S.) was

TABLE I

RELATIVE MOLAR RESPONSES (RMR) OF N(O)-*tert.*-BUTYLDIMETHYLSILYLAMINO ACID DERIVATIVES

Amino acid	RMR[a]	S.D.[b]	R.S.D. (%)[b]
Ala	2.855	0.113	3.96
Gly	2.620	0.083	3.17
Val	3.190	0.015	3.61
Leu	3.343	0.108	3.23
Ile	3.293	0.155	4.71
Thr	1.178	0.058	4.92
Gln	2.430	0.048	1.98
Met	2.920	0.068	2.33
Ser	1.675	0.075	4.48
Pro	1.895	0.068	3.59
Phe	3.298	0.055	1.67
Asp	3.353	0.053	1.58
Hyp1	0.885	0.008	0.90
Cys	0.648	0.030	4.63
Glu	2.665	0.088	3.30
Asn	1.898	0.078	4.11
Lys	1.670	0.075	4.49
Hyp2	0.758	0.037	4.88
Arg	0.670	0.032	4.78
His	0.815	0.008	0.98
Trp	1.928	0.060	3.11
Tyr	3.148	0.140	4.45
Cyt	0.410	0.020	4.88

[a] Values are relative to pyrene = 1.
[b] $n = 4$.

750 pmol. The relative standard deviations (R.S.D.s) were less than 5% ($n = 4$) for all of the amino acid derivatives. The reproducibility showed high precision. The arginine, hydroxyproline, histidine, cysteine and cystine peaks were relatively small; especially the cystine peak was so small that it could not be detected at levels below 400 pmol. There are several reports that arginine is the most difficult amino acid to derivatize completely [7,8,10], but in our study cystine also seemed to be difficult to derivatize at temperatures below 75°C.

Calibration graphs for all of the amino acid derivatives, obtained by plotting the ratios of their peak areas to that of internal standard, showed good linearity in the range 238–833 pmol, except for the cystine derivative, which showed linearity in the range 476–1900 pmol (Table II). Cystine, proline, histidine and arginine showed poor linearity compared with the other derivatives. The correlation coefficients of the calibration graphs for these derivatives were also highly significant ($p < 0.001$). Typically, the R.S.D.s were lower than 5% ($n = 5$), except for serine, proline, cysteine, arginine, histidine, tryptophan and cystine, which showed higher values but <10%.

Mass spectra

The identities of the derivatives were established by EI mass spectrometry. The mass spectra were characterized by the ions M − 15 (CH_3), M − 57 [$C(CH_3)_3$], M − 85 [$C(CH_3)_3$ + CO], M − 159 [$COOSi(CH_3)_2C(CH_3)_3$], M − R, *m/z* 189 [$(CH_3)C(CH_3)_2\overset{+}{S}iO = Si(CH_3)_2$], 147 [$(CH_3)_3\overset{+}{S}iO = Si(CH_3)_2$], 133 [$(CH_3)_2H\overset{+}{S}iO = Si(CH_3)_2$] and 73 [$(CH_3)_3\overset{+}{S}i$]. In sulphur amino acids, additional fragmentations were observed for C–S and S–S bond cleavage with charge retention on either fragment. These fragmentation patterns were similar to those for TMS derivatives [16] and tBDMSi derivatives in other reports [7,8,10,11], where TMS derivatives of aliphatic α-amino acids were characterized by molecular ions M − 15 (CH_3), M − 43 (CH_3 + CO), M − 117 (COOTMS) and M − R [16] and tBDMSi amino acid derivatives by molecular ions, M − 15, M − 57, M − 85, M − 159, M − 302 and M − R [7,10].

TABLE II

LINEAR REGRESSION ANALYSIS OF RELATIVE MOLAR RESPONSE AGAINST AMOUNT INJECTED OF AMINO ACID AS THEIR tBDMSi DERIVATIVES

Internal standard, pyrene: amounts injected, 238, 357, 476, 595, 714 and 833 pmol ($n = 4$).

Amino acid	Regression line[a]		
	s	*b*	*r*
Alanine	0.0017	0.081	0.979
Glycine	0.0017	0.022	0.989
Valine	0.0017	0.153	0.992
Leucine	0.0019	0.129	0.991
Isoleucine	0.0019	0.116	0.994
Threonine	0.0009	−0.108	0.992
Glutamine	0.0014	0.097	0.983
Methionine	0.0018	0.052	0.991
Serine	0.0014	−0.060	0.969
Proline	0.0011	0.002	0.935
Phenylalanine	0.0021	0.018	0.989
Aspartic acid	0.0022	0.030	0.993
Cysteine	0.0007	−0.073	0.979
Glutamic acid	0.0019	−0.008	0.991
Asparagine	0.0014	−0.072	0.989
Lysine	0.0014	−0.164	0.992
2-Hydroxyproline	0.0005	0.056	0.968
Arginine	0.0005	0.007	0.964
Histidine	0.0011	−0.342	0.975
Tryptophan	0.0018	−0.338	0.982
Tyrosine	0.0024	−0.102	0.992
Cystine	0.0011	−0.060	0.969

[a] s = Slope; b = intercept; r = correlation coefficient.

The relative intensity of the major ions in the EI mass spectra of tBDMSi amino acid derivatives are given in Table III. Molecular ions were not detected except for methionine, lysine and histidine. The relative intensity of the lysine molecular ion was high (7.1%). The fragmented ions of M − 57, M − 85 and *m/z* 147 and 73 were detected with high intensity for all derivatives. Even though the fragment of *m/z* 133 was detected with relatively lower intensity than the above fragments, it could be observed in all derivatives.

The *m/z* 133 fragment was also observed in the chemical ionization mass spectra of the tBDMSi derivatives of glutamine and asparagine in another study [5]. In our study, we considered

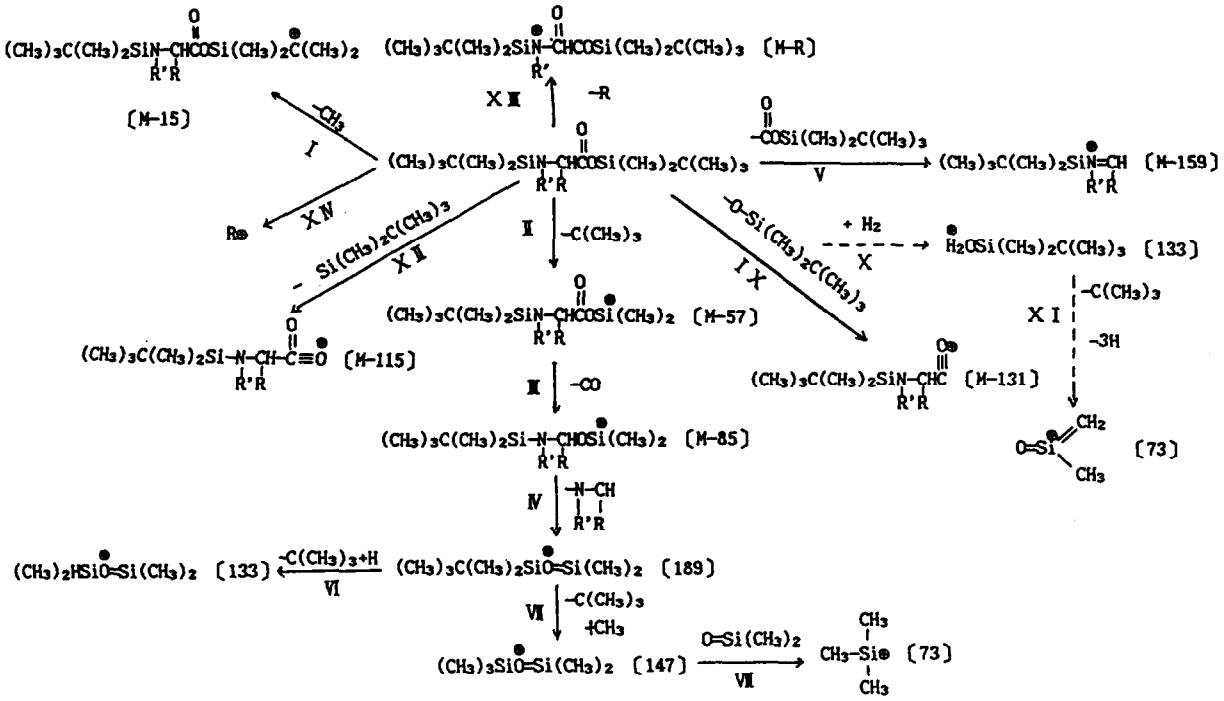

Fig. 2. Possible MS fragmentation pathways of N(O)-*tert.*-butyldimethylsilylamino acids.

that the *m/z* 133 fragment would be produced from pathway VI (Fig. 2), as an intermediate fragment of *m/z* 189 $[(CH_3)_3C(CH_3)_2SiO{=}Si(CH_3)_2]$ prior to the *m/z* 133 fragment was detected for all the derivatives (Table III). If the *m/z* 133 fragment were $[H_2OSi(CH_3)_2C(CH_3)_3]$, the M − 131 fragment should be detected for all the derivatives, but it was not observed with most of them. We therefore consider that pathways IX, X and XI (Fig. 2) hardly take place. The intensity of the M − 57 fragment was far greater than that of the M − 15 fragment. As neutral radicals have the stability order tertiary > secondary > primary [17], the cleavage pathway would favour pathway II rather than I (Fig. 2).

Mass spectral fragmentations of tBDMSi amino acids are shown in Figs. 3–7. All the derivatives were broken down almost according to the pathways in Fig. 2. The glutamine mass spectrum was consistent with the conversion of glutamine to pyroglutamic acid. It could be concluded, on the basis of the mass spectrum, that the glutamine derivative was a pyroglutamic acid derivative that had been quantitatively converted into pyroglutamic acid during the derivatization reaction [5]. However, glutamine is converted to ammonia and pyroglutamic acid on heating under weakly acidic or alkaline conditions [18,19] and the reaction proceeds slowly even at *ca.* 4°C. Further, there is a report that glutamine can be derivatized with MTBSTFA without loss of the amino moiety and without cyclization to produce a chromatographically stable compound [11]. It appears, therefore, that the conversion to pyroglutamic acid occurred during the storage of standard glutamine in 0.1 *M* hydrochloric acid.

An M − 13 fragment was found for proline and hydroxyproline. We assume that this fragment was produced as follows:

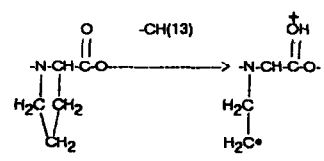

TABLE III

RELATIVE INTENSITIES OF MAJOR IONS IN THE EI MASS SPECTRA OF N(O)-*tert.*-BUTYLDIMETHYLSILYL (tBDMSi) AMINO ACID DERIVATIVES

Ion	Ala	Gly	Val	Leu	Ile	Thr	Gln	Met	Ser	Pro	Phe	Asp	Hyp1	Cys	Glu	Asn	Lys	Hyp2	Arg	His	Trp	Tyr	Cyt
M	–	–	–	–	–	–	–	1.4	–	–	–	–	–	–	–	–	7.1	–	–	0.4	–	–	–
$M - CH_3$	2.8	3.2	2.2	1.8	1.9	1.4	7.5	3.0	4.7	0.4	3.1	2.0	0.4	2.5	3.2	1.5	1.2	–	–	2.1	–	2.3	–
$M - C(CH_3)_3$	55.3	77.3	36.6	36.8	52.9	53.4	100	75.1	75.6	5.7	49.5	45.2	26.0	63.0	86.1	90.1	13.7	5.7	–	44.4	28.9	9.5	3.3
$M - C(CH_3)_3 - CO$	56.3	74.3	40.4	37.3	37.9	5.4	79.3	83.9	67.8	20.1	45.6	20.5	17.7	54.4	9.6	–	–	8.8	–	1.7	5.1	4.5	–
M – COOtBDMSi	100	17.2	100	100	100	29.4	21.1	100	87.5	27.5	66.7	35.0	22.0	84.8	72.3	12.3	17.8	30.7	2.4	30.6	6.6	9.1	1.6
M – R	–	–	15.8	36.8	52.9	42.5	–	4.7	46.3	–	100	69.2	–	88.5	–	33.6	66.4	–	4.8	8.7	100	100	47.8
R^+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8.4	100	24.7	5.0	–
$(CH_3)_3C(\overset{+}{C}H_3)_2SiO = Si(CH_3)_2$	1.8	8.0	1.3	1.0	1.7	6.6	2.4	2.4	2.1	0.5	1.3	4.7	1.0	1.5	2.6	4.4	1.5	1.0	7.1	0.2	2.0	1.0	7.7
$(CH_3)_3SiO = Si(CH_3)_2$	45.5	100	23.0	18.2	19.5	25.9	15.0	46.7	31.8	14.7	29.7	27.3	19.8	25.9	35.9	30.4	23.8	23.8	14.5	5.5	9.9	6.8	19.7
$HSi(CH_3)_2O = Si(CH_3)_2$	8.1	9.3	6.1	5.3	6.2	10.7	8.3	10.9	14.6	4.6	9.2	10.7	7.3	10.5	11.9	9.7	10.9	11.3	7.2	2.4	6.6	2.3	8.0
$(CH_3)_3Si^+$	54.2	73.8	40.6	33.2	42.2	100	79.4	85.7	100	39.8	68.6	100	72.6	100	100	100	100	92.3	100	53.3	57.4	40.9	100

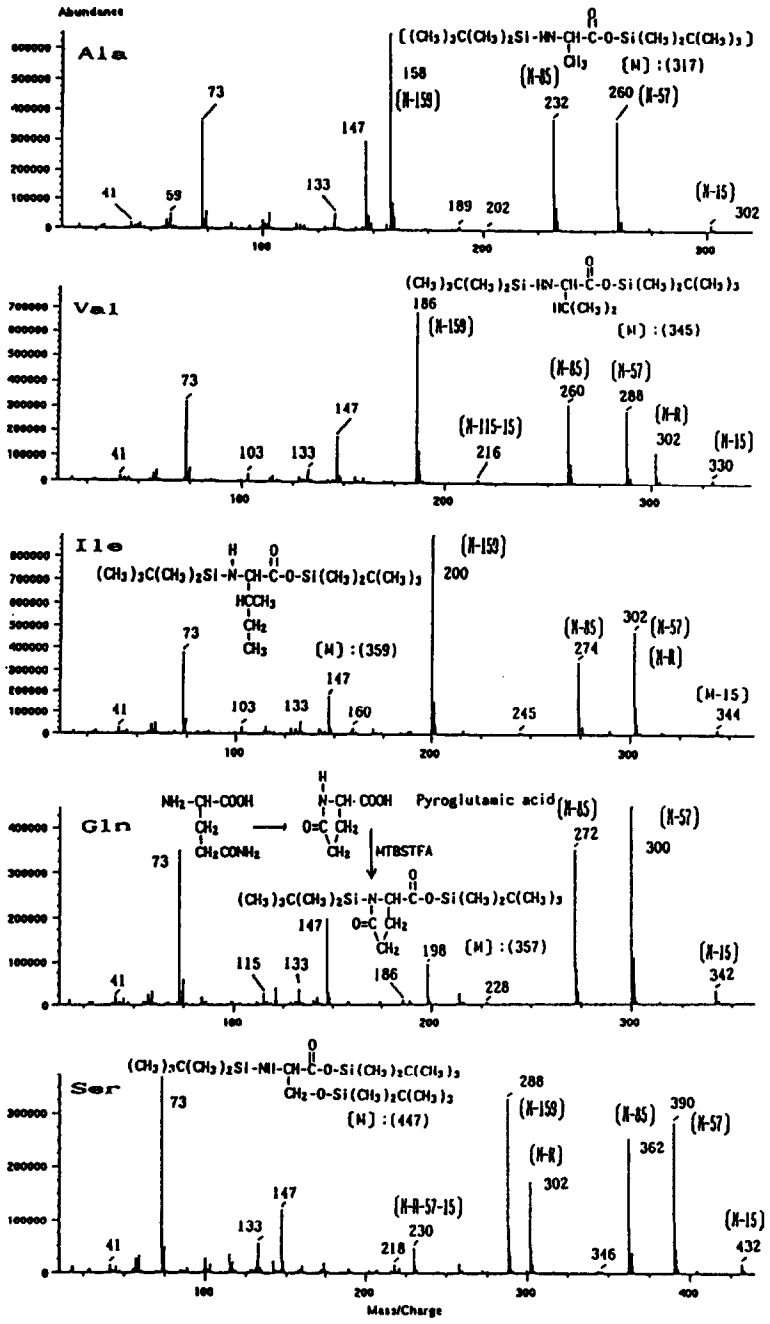

Fig. 3. EI mass spectra of N(O)-*tert.*-butyldimethylsilyl derivatives of alanine, valine, isoleucine, glutamine and serine. M − 15, CH_3; M − 57, $C(CH_3)_3$; M − 85, $C(CH_3)_3 + CO$, M − 115, $Si(CH_3)_2C(CH_3)_3$; M − 131, $OSi(CH_3)_2C(CH_3)_3$; M − 159, $COOSi(CH_3)_2C(CH_3)_3$; 189, $(CH_3)_3C(CH_3)_2SiO = Si(CH_3)_2$; 147, $(CH_3)_3Si\text{-}O = Si(CH_3)_2$; 133, $(CH_3)_2HSiO = Si(CH_3)_2$; 73, $(CH_3)_3Si$.

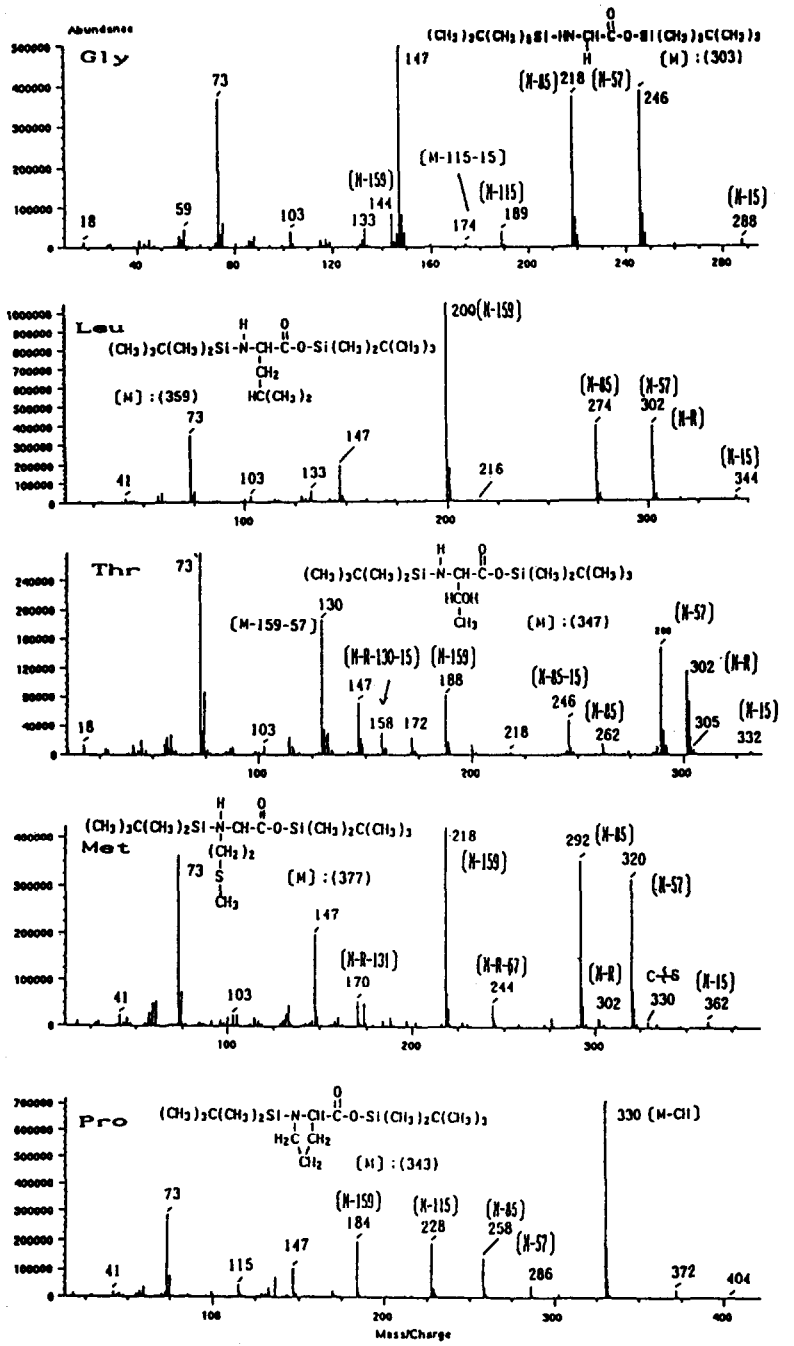

Fig. 4. EI mass spectra of N(O)-*tert.*-butyldimethylsilyl derivatives of glycine, leucine, threonine, methionine and proline.

In the spectrum of arginine, ions of m/z 442 ($M - C_4H_7 - CH_3$), 340 ($M - 172$) and 207 were found with other unusual ions. In the spectrum of cystine, the M/2 ion produced from cleavage of the S–S bond was observed with high intensity.

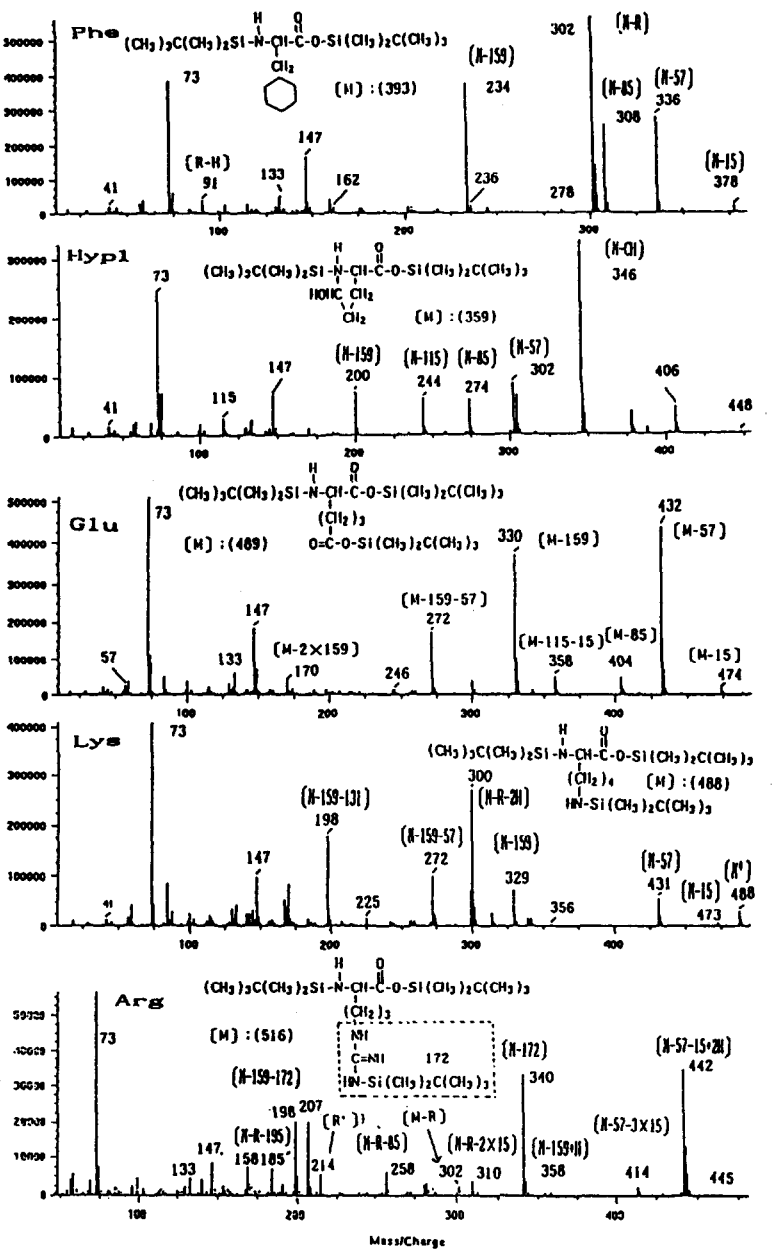

Fig. 5. EI mass spectra of N(O)-*tert.*-butyldimethylsilyl derivatives of phenylalanine, hydroxyproline-1, glutamic acid, lysine and arginine.

CONCLUSIONS

Amino and carboxyl groups of all protein amino acids can be silylated to the N(O)-*tert.*-butyldimethylsilyl derivatives by a single-step reaction with N-methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide as the silylating reagent.

All 22 protein amino acid derivatives were

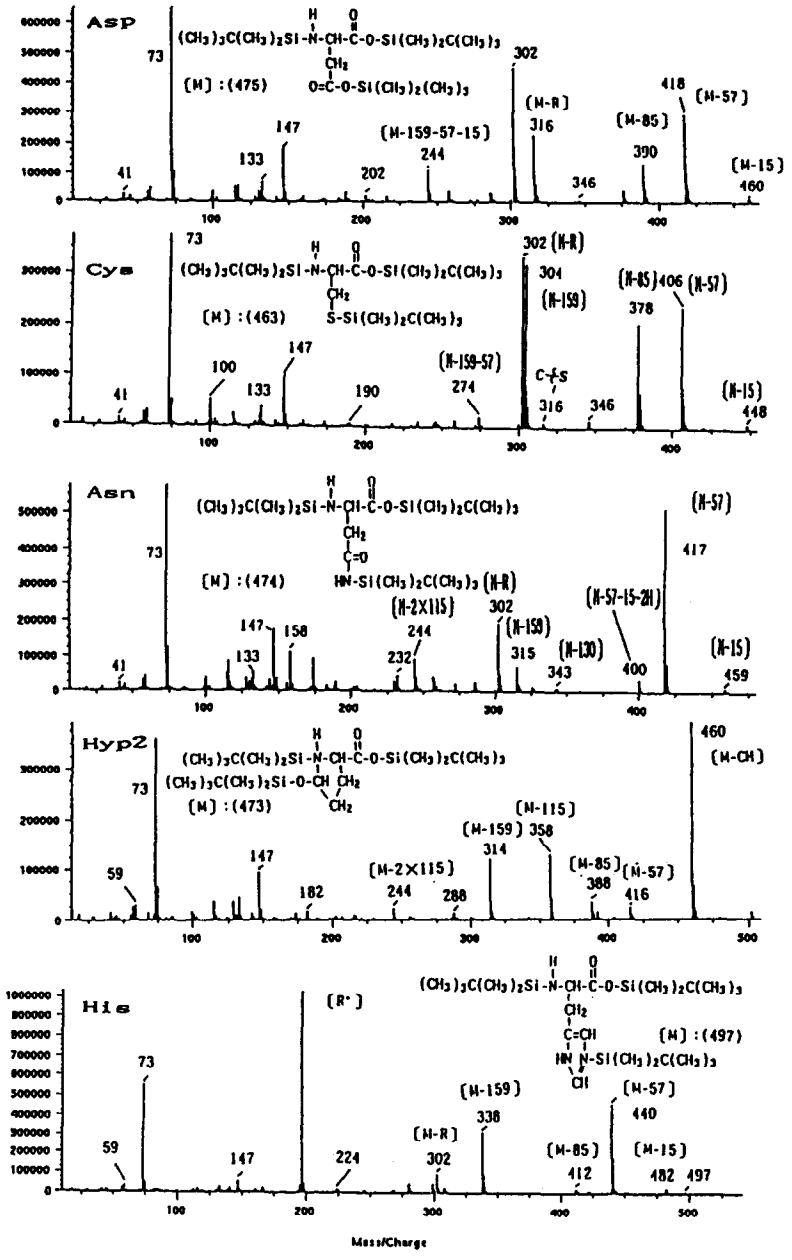

Fig. 6. EI mass spectra of N(O)-*tert.*-butyldimethylsilyl derivatives of aspartic acid, cysteine, asparagine, hydroxyproline-2 and histidine.

successfully separated on a DB-1 capillary column (60 m × 0.35 mm I.D.). Because the R.S.D.s of the relative molar responses were <5% and the calibration graphs showed good linearity, this method could be useful for both the qualitative and quantitative analysis of protein amino acids. The extension of this method to biological amino acids is in progress.

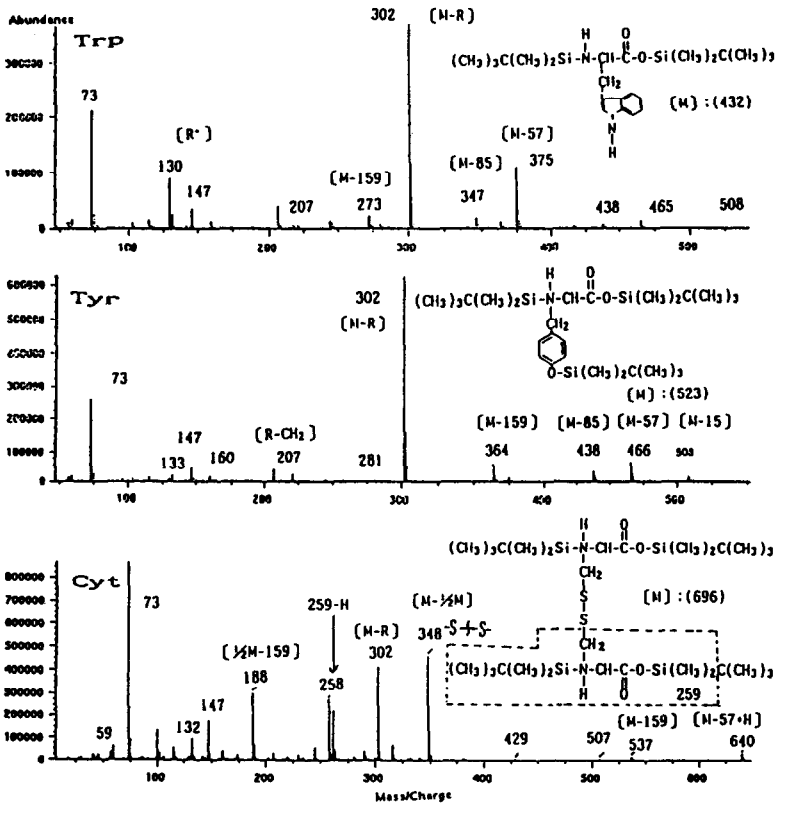

Fig. 7. EI mass spectra of N(O)-*tert.*-butyldimethylsilyl derivatives of tryptophan, tyrosine and cystine.

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