Journal of Chromatography, 605 (1992) 241–249
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 229

Capillary gas chromatography of protein amino acids as N(O,S)-isobutyloxycarbonyl *tert.*-butyldimethylsilyl derivatives in aqueous samples

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(First received September 23rd, 1991; revised manuscript received March 31st, 1992)

ABSTRACT

A method for the quantitative determination of the nineteen protein amino acids by gas chromatography is described. The amino acids were allowed to react with isobutyl chloroformate in a basic aqueous medium and the resulting N(O,S)-isobutyloxycarbonyl (isoBOC) amino acids were extracted into an organic solvent after acidification. The isolated N(O,S)-isoBOC amino acids were then converted to stable *tert.*-butyldimethylsilyl (TBDMS) derivatives, which were analyzed by gas chromatography and gas chromatography–mass spectrometry. The characteristic [M − 57], [M − 113], [M − 159], [M − 174], etc. ions in the mass spectra of N(O,S)-isoBOC TBDMS derivatives enabled rapid confirmation of the amino acids. Temperature-programmed retention index (I) sets measured on DB-5 and DB-17 capillary columns were characteristic of each amino acid thus being useful for the quick identification by computer I matching. The overall extraction and derivatization yields of the amino acids studied were linear in the range of 20–80 μg.

INTRODUCTION

High-resolution capillary gas chromatography (GC) of amino acids requires blocking of the active protons on the amino, carboxyl, hydroxyl and thiol groups. The preparation of volatile, yet stable derivatives by a single-step procedure is most desirable and the silylation is the closest approach to this goal. When the trimethylsilyl (TMS) function was used for the silylation of amino acids, the inherent instability of the resulting TMS derivatives toward hydrolysis and the excessive silylation of the nitrogen atoms led to the formation of multiple derivatives for some amino acids [1–3].

The *tert.*-butyldimethylsilyl (TBDMS) function first employed as a more moisture stable alternative to the TMS group [4] is now widely used for the silylation of hydroxyl and carboxyl groups using N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [5] as the silylating reagent. The TBDMS derivatives were found to have superior GC and mass spectral (MS) properties [6,7]. In a previous report, we demonstrated that multifunctional organic acids were quantitatively converted to their TBDMS derivatives, yielding a single

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0021-9673/92/$05.00

peak for each organic acid [8]. In recent years, the TBDMS derivatization has been successfully extended to amino acids [9–16]. However, a painstaking step for the moisture removal from the hydrochloride salts of amino acids is prerequisite.

Prior to conversion to suitable volatile derivatives, amino acids are isolated from complex aqueous samples mainly by the multi-step ion-exchange technique though its inherent drawbacks are well known [17]. There appears to be a need for improvement of sample purification. In the literature, as a different approach, Makita and co-workers [18–21] demonstrated that amino acids were quantitatively extracted with diethyl ether from aqueous media after selective blocking of the active hydrogens on the amino, thiol, imidazole, and phenolic hydroxyl groups by reaction with isobutyl chloroformate (isoBCF). The remaining carboxyl groups of the resulting N(O,S)-isobutyloxycarbonyl (-isoBOC) amino acids were then methylated with diazomethane.

It occurred to us that by combining Makita's N(O,S)-isoBOC procedure for the selective purification of amino acids from aqueous samples and the TBDMS derivatization of the carboxyl and remaining amide or alcoholic hydroxyl groups in the N(O,S)-isoBOC amino acids would give a better result for the GC analysis of amino acids. The present work describes a method for the GC analysis of amino acids in aqueous samples as their N(O,S)-isoBOC TBDMS derivatives. The structures of the derivatives were confirmed by GC–MS.

EXPERIMENTAL

Materials

All of the amino acids used in this study, isobutyl chloroformate (isoBCF), and *tert.*-butyldimethylchlorosilane (TBDMCS) were purchased from Sigma (St. Louis, MO, USA); dimethylformamide (DMF), acetonitrile, pyridine and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) of silylation grade from Pierce (Rockford, IL, USA). Diethyl ether purchased from Oriental Chemical Industry (Seoul, Korea) was distilled over potassium hydroxide in an all-glass apparatus and stored in an amber screw-cap bottle in the presence of anhydrous sodium sulfate at 4°C. Sodium chloride was purchased from Junsei (Tokyo, Japan), sodium carbonate and hydrogen chloride from Duksan (Seoul, Korea), and *n*-hydrocarbon standards (C_{12}–C_{36}, even numbers only) from Polyscience (Niles, IL, USA).

Amino acid solutions

The amino acid solution containing nineteen amino acids, 0.5 μg each per μl in 0.1 *M* HCl was used as the working solution. Arginine was excluded from this study. 3,4-Dimethoxybenzoic acid (Aldrich, Milwaukee, USA) used as the internal standard was dissolved in methanol at a concentration of 5 μg/μl.

N(O,S)-isoButyloxycarbonylation of amino acids

According to the procedure of Makita *et al.* [18], the internal standard solution (20 μg), distilled water (1.5 ml) and 5% sodium carbonate solution (0.5 ml) and isoBCF (0.1 ml) were added to an aliquot (100 μl) of the amino acid solution. The mixture was shaken for 10 min at room temperature and washed with diethyl ether (4 × 2 ml). The aqueous mixture was then adjusted to pH 1–2 (10% hydrochloric acid), saturated with sodium chloride, and was extracted with diethyl ether (4 × 1 ml).

tert.-Butyldimethylsilylation of N(O,S)-isoBOC amino acids

The ether extract containing N(O,S)-isoBOC amino acids was evaporated to dryness under gentle stream of nitrogen at 50°C. To the residue were added 10 μl of acetonitrile and 50 μl of MTBSTFA. The mixture was heated to 60°C for 20 min to form TBDMS derivatives. The reaction mixture was directly examined by GC and GC–MS. The calibration samples for precision tests of the overall procedure were prepared with amino acid solutions containing 20, 40 or 80 μg of each amino acid and 20 μg of internal standard. In place of acetonitrile, dimethylformamide and pyridine were investigated as solvent and also 1% TBDMCS in the MTBSTFA as catalyst. The effect of varying temperature (room temperature to 90°C) and heating time on the derivative yields was also investigated.

Gas chromatography

GC analyses were performed with Hewlett-Packard HP 5890A gas chromatograph, equipped with a split/splitless capillary inlet system, and two

flame ionization detectors, an HP 3392A integrator, and interfaced to an HP 5895A GC Chemstation on a 25 m × 0.32 mm I.D. fused-silica Ultra-1 capillary column (film thickness, 0.17 μm; Hewlett-Packard, Avondale, PA, USA). Nitrogen at a flow-rate of 1.03 ml/min was used as the carrier gas, and *ca.* 1-μl aliquots of samples were injected with a split ratio of 30:1. After an initial hold time of 2 min at 120°C, oven temperature was programmed to 280°C at a rate of 5°C/min. The injector and detector temperatures were 280 and 300°C, respectively. DB-5 (30 m × 0.25 mm I.D., 0.24 μm d_f) and DB-17 (30 m × 0.25 mm I.D., 0.25 μm d_f) fused-silica capillary columns (J. & W. Scientific, Rancho Cordova, CA, USA) were used for the retention index (*I*) measurement. A DB-1701 (30 m × 0.25 mm I.D., 0.25 μm d_f) fused-silica capillary column was also investigated as a potential column for *I* measurement. For the *I* measurement, after an initial hold time of 2 min at 150°C, oven temperature was programmed to 280°C at a rate of 3°C/min. A standard solution of *n*-hydrocarbons (C_{12}–C_{36}, even numbers only) in isooctane was co-injected with the samples in the split mode (30:1). Samples were analyzed in triplicate.

Gas chromatography–mass spectrometry

A Hewlett-Packard HP 5890A gas chromatograph, interfaced to an HP 5970B MSD (70 eV, EI mode) which was on-line to a HP 59940A MS Chemstation system, was used with an HP-1 cross-linked capillary column (12 m × 0.20 mm I.D., 0.33 μm film thickness) to obtain mass spectra. Samples were introduced in the split-injection mode (30:1) at 260°C, and the oven temperature was initially 100°C then programmed to 280°C at a rate of 15°C/min. The interface temperature was 300°C. The mass range scanned was 60–500 a.m.u. at a rate of 1.29 scan/s.

RESULTS AND DISCUSSION

As an alternative to the conventional ion-exchange clean-up for protein amino acids from aqueous samples prior to the TBDMS derivatization, we adopted Makita's N(O,S)-isobutyloxycarbonyl (-isoBOC) reaction method [18] with minor modifications. Prior to extraction with diethyl ether, the reaction mixture was acidified by HCl solution rather than H_3PO_4 since the large TBDMS triester of the extracted H_3PO_4 interfered in the GC analysis of the amino acid derivatives. The N(O,S)-isoBOC reaction readily transforms the zwitterionic amino acids into the corresponding organic extractable carboxylic acids by blocking the basic amino functions with isoBCF in a basic aqueous medium. Thiol, phenolic hydroxyl and imidazole functions, if present, are blocked too. Arginine was excluded from the present study as it was shown not to be amenable directly to the Makita method [18].

The TBDMS derivatization of the resulting N(O,S)-isoBOC amino acids offers advantages over the Makita methylation [18], since polar hydroxyl and amide functions as well as the carboxyl group are converted to TBDMS derivatives which generate diagnostically useful [M − 57] ions in their mass spectra. With the exception of glutamine, asparagine, serine and threonine, the TBDMS derivatization of the N(O,S)-isoBOC amino acids in acetonitrile (10 μl) was completed when mixed with MTBSTFA (50 μl) at room temperature. Serine with a hydroxyl function required longer reaction time at room temperature for complete conversion to di-TBDMS derivative. Threonine, due to the steric hindrance of the β-hydroxyl group toward the bulky TBDMS moiety, gave mono- and di-TBDMS derivatives even after extended heating at 60°C. Basic amino acids such as lysine, asparagine and glutamine when heated at 90°C or higher produced diminished responses. At a higher temperature and with extended heating the responses of all the amino acid derivatives generally were reduced, especially those of alanine and glycine. The addition of 1% (w/w) TBDMCS to MTBSTFA did not improve the derivative yields.

When the reaction in DMF was conducted at 75°C for 2 h, the responses of amino acids except for proline were significantly reduced and also in pyridine, cysteine, lysine, histidine and tyrosine were observed to give smaller responses. On the other hand, when the reaction was performed directly in MTBSTFA without any added solvent, good results were produced although the stability of the derivatives was not very good.

In general, the condition producing optimal overall derivatization was heating at 60°C for 15 min in acetonitrile and MTBSTFA.

The three combined procedures including N(O,

S)-isoBOC reaction, solvent extraction and TBDMS derivatization were examined for the overall precision and linearity of the calibration plots. A linear response in the range of 20–80 μg was obtained for most amino acids with the correlation coefficients approaching 0.99 as listed in Table I. For serine and threonine, each combined peak area of mono- and di-TBDMS peaks was used. Typically, the relative standard deviation ($n = 3$) was lower than 5% on the average except for lysine, histidine, tryptophan and tyrosine which gave higher R.S.D. but within 8%. Considering the method consists of three independent steps, its overall reproducibility is satisfactory for the quantitation of amino acids.

The separation of the nineteen amino acid derivatives on three different fused-silica capillary columns is presented in Fig. 1. Each amino acid derivative displayed a single symmetrical peak with the exception of serine and threonine which exhibited two peaks due to mono- and di-TBDMS derivatives, labeled serine-1, serine-2, threonine-1, and threonine-2, respectively. Serine-2 and threonine-2 were not adequately resolved on Ultra-1, DB-5 and DB-17 columns. Temperature-programmed retention index sets for each derivative measured on DB-5 and DB-17 columns are given in Table II. The *I* sets were characteristic of each amino acid, thus being useful for the quick identification by the computer *I* library matching as described in our organic acid profiling works [22,23].

When a DB-1701 column was used, no peaks were obtained from the derivatives of glutamine and asparagine. Their labile N-TBDMS bonds appear to be decomposed by the polar cyanopropyl functions of the DB-1701 column.

Most N(O,S)-isoBOC TBDMS derivatives of

TABLE I

LINEAR REGRESSION ANALYSIS OF RELATIVE RESPONSE AGAINST RELATIVE WEIGHT OF AMINO ACID AS THEIR N(O,S)-isoBOC TBDMS DERIVATIVES

Amino acid	Mean AR ± S.D. (%R.S.D.)[a]			Regression line		
	20 μg	40 μg	80 μg	m[b]	b[c]	r[d]
Alanine	1.610 ± 0.059 (3.7)	3.610 ± 0.074 (2.0)	7.358 ± 0.194 (2.6)	1.910	−0.264	0.999
Glycine	1.705 ± 0.071 (4.2)	3.929 ± 0.067 (1.7)	7.769 ± 0.133 (1.7)	2.007	−0.215	0.999
Valine	1.497 ± 0.034 (2.3)	3.309 ± 0.033 (1.0)	6.550 ± 0.114 (1.7)	1.675	−0.124	0.999
Leucine	1.332 ± 0.026 (2.0)	2.892 ± 0.056 (1.9)	5.607 ± 0.163 (2.9)	1.415	−0.026	0.999
Isoleucine	1.303 ± 0.018 (1.4)	2.808 ± 0.043 (1.5)	5.475 ± 0.111 (2.0)	1.383	−0.031	0.999
Proline	1.501 ± 0.006 (0.4)	3.197 ± 0.028 (0.9)	6.159 ± 0.038 (0.6)	1.542	0.020	0.999
Methionine	0.872 ± 0.038 (4.4)	1.942 ± 0.078 (4.0)	3.835 ± 0.183 (4.8)	0.982	−0.075	0.999
Serine	1.319 ± 0.031 (2.4)	2.775 ± 0.032 (1.2)	4.966 ± 0.240 (4.8)	1.199	0.224	0.997
Threonine	0.648 ± 0.021 (3.2)	1.421 ± 0.031 (2.2)	2.731 ± 0.121 (4.4)	0.688	−0.007	0.999
Phenylalanine	1.385 ± 0.021 (1.5)	2.556 ± 0.126 (4.9)	5.172 ± 0.211 (4.1)	1.269	0.077	0.999
Aspartic acid	1.538 ± 0.043 (2.8)	2.853 ± 0.095 (3.3)	5.600 ± 0.238 (4.3)	1.357	0.165	0.999
Cysteine	1.118 ± 0.053 (4.7)	2.315 ± 0.116 (5.0)	4.289 ± 0.212 (4.9)	1.047	0.131	0.999
Glutamic acid	0.714 ± 0.021 (2.9)	1.237 ± 0.056 (4.5)	2.432 ± 0.096 (3.9)	0.576	0.117	0.999
Asparagine	0.175 ± 0.007 (4.0)	0.432 ± 0.021 (4.9)	0.846 ± 0.036 (4.3)	0.221	−0.032	0.998
Glutamine	0.345 ± 0.015 (4.3)	0.579 ± 0.027 (4.7)	1.257 ± 0.006 (0.5)	0.309	0.006	0.996
Lysine	0.519 ± 0.007 (1.3)	0.851 ± 0.037 (4.3)	1.794 ± 0.121 (6.7)	0.432	0.048	0.997
Histidine	0.213 ± 0.012 (5.6)	0.458 ± 0.034 (7.4)	0.826 ± 0.058 (7.0)	0.201	0.029	0.997
Tryptophan	0.869 ± 0.026 (3.0)	1.354 ± 0.084 (6.2)	2.862 ± 0.106 (3.7)	0.677	0.115	0.995
Tyrosine	0.185 ± 0.014 (7.6)	0.603 ± 0.032 (5.3)	1.098 ± 0.087 (7.9)	0.296	−0.063	0.990

[a] AR = Peak area ratio relative to internal standard (I.S.); S.D. = standard deviation for $n = 3$.
[b] m = Slope = relative weight response = mean AR × weight of I.S./weight of amino acid.
[c] b = y-intercept.
[d] Correlation coefficient.

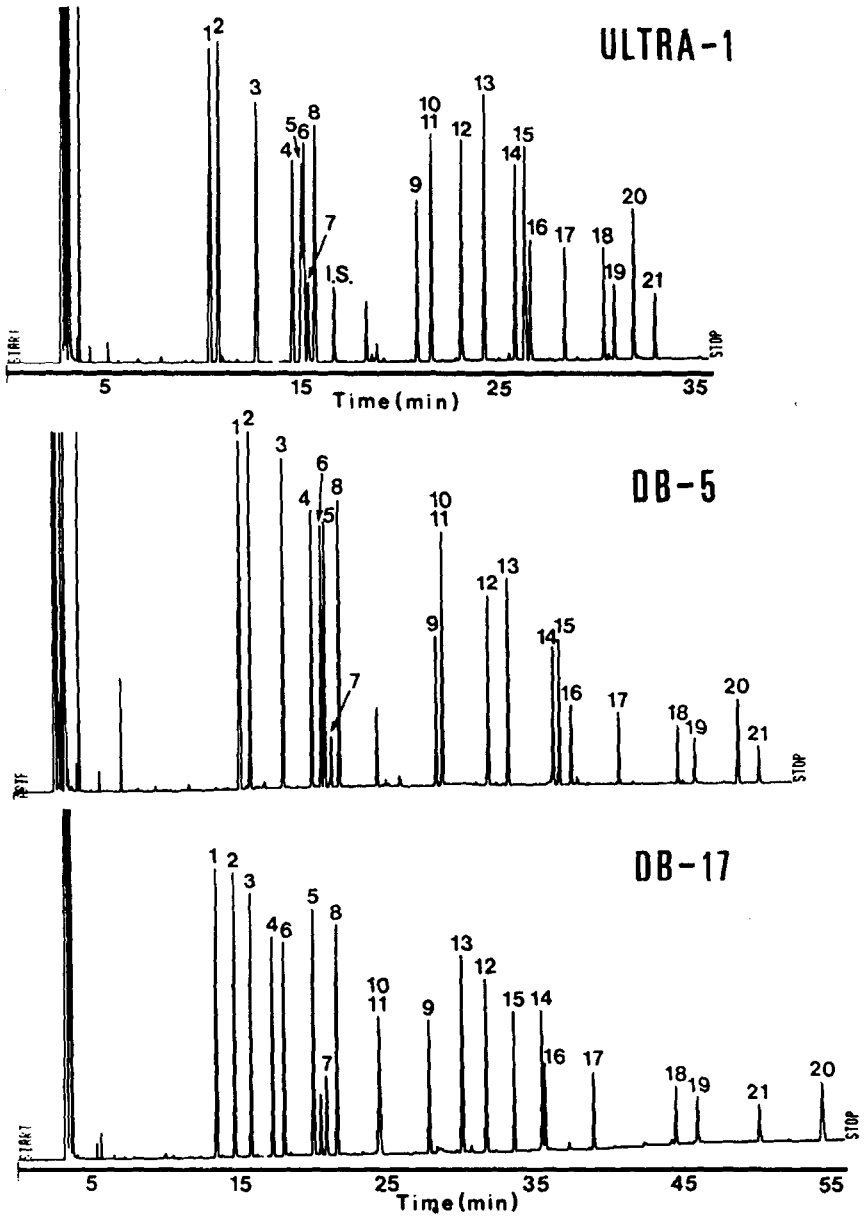

Fig. 1. Chromatograms of the nineteen amino acid mixture as their N(O,S)-isoBOC TBDMS derivatives separated on Ultra-1 (25 m × 0.32 mm I.D.), DB-5 (30 m × 0.25 mm I.D.) and DB-17 (30 m × 0.25 mm I.D.) fused-silica capillary columns. GC conditions are in the text. Peaks: 1 = alanine; 2 = glycine; 3 = valine; 4 = leucine; 5 = threonine-1; 6 = isoleucine; 7 = serine-1; 8 = proline; 9 = methionine; 10 = serine-2; 11 = threonine-2; 12 = phenylalanine; 13 = aspartic acid; 14 = cystein; 15 = glutamic acid; 16 = asparagine; 17 = glutamine; 18 = lysine; 19 = histidine; 20 = tryptophan; 21 = tyrosine (peak numbers correspond to those in Table II).

TABLE II

GAS CHROMATOGRAPHIC AND MASS SPECTRAL DATA OF N(O,S)-isoBOC TBDMS DERIVATIVES OF AMINO ACIDS

Amino acid	GC I^a data set		Mol. wt.	Mass spectral data set						
	DB-5	DB-17		[M−57]	[M−15]	[M−113]	[M−131]	[M−159]	[M−174]	Other ion
1 Ala	1702.2	1854.8	303	246 (33)	288 (1)	190 (100)	172 (2)	144 (23)	129 (2)	
2 Gly	1724.9	1901.1	289	232 (26)	274 (0)	176 (100)	158 (8)	130 (4)		
3 Val	1800.0	1942.9	331	274 (35)	316 (1)	218 (100)	200 (2)	172 (28)	157 (3)	
4 Leu	1860.1	2000.0	345	288 (38)	330 (1)	232 (100)	214 (2)	186 (36)	171 (3)	
5 Thr-1	1887.3	2094.6	333	276 (11)	318 (1)	220 (64)	202 (100)	174 (9)	159 (4)	
6 Ile	1879.8	2026.7	345	288 (39)	330 (1)	232 (100)	214 (2)	186 (35)	171 (3)	
7 Ser-1	1900.8	2127.8	319	262 (12)	304 (1)	206 (46)	188 (100)	160 (15)	145 (5)	
8 Pro	1917.9	2152.0	329	272 (50)	314 (1)	216 (100)	198 (0)	170 (56)	155 (0)	
9 Met	2133.5	2372.1	363	306 (65)	348 (3)	250 (40)	232 (28)	204 (21)	189 (5)	178 (100)
10 Ser-2	2146.8	2252.7	433	376 (100)	418 (4)	320 (14)	302 (33)	274 (11)	259 (4)	
11 Thr-2	2146.8	2255.2	447	390 (100)	432 (4)	334 (7)	316 (28)	288 (17)	273 (5)	
12 Phe	2251.9	2515.6	379	322 (82)	364 (2)	266 (100)	248 (14)	220 (18)	205 (57)	
13 Asp	2299.0	2454.9	461	404 (100)	446 (2)	348 (3)	330 (12)	302 (15)	287 (49)	
14 Cys	2405.8	2663.7	435	378 (100)	420 (3)	322 (23)	304 (6)	276 (16)	261 (25)	
15 Glu	2421.2	2588.8	475	418 (100)	460 (3)	362 (2)	344 (89)	316 (58)	301 (2)	
16 Asn	2450.9	2671.6	460	403 (100)	445 (5)	347 (4)	329 (19)	301 (3)	286 (27)	
17 Gln	2572.3	2791.0	474	417 (100)	459 (4)	361 (2)	343 (33)	315 (24)	300 (2)	
18 Lys	2729.0	3050.2	460	403 (44)	445 (3)	347 (6)	329 (15)	301 (2)	286 (0)	184 (100)
19 His	2772.6	3111.4	469	412 (93)	454 (9)	356 (3)	338 (42)	310 (91)	295 (100)	
20 Trp	2866.9	3404.8	418	361 (8)	403 (1)	305 (1)	287 (4)	259 (3)	244 (23)	130 (100)
21 Tyr	2911.5	3269.2	495	438 (12)	480 (1)	382 (9)	364 (6)	336 (3)	321 (100)	

[a] R.S.D. ranged from 0.01–0.05% from three measurements.

amino acids were stable for at least two months at 4°C kept in tightly closed Teflon-lined screw-cap reaction vials, indicating that they are more stable than the corresponding N(O,S,COO)-TBDMS derivatives [9–15].

The amino acid derivatives were subjected to GC–MS analysis. The electron-impact MS data are summarized in Table II. As is characteristic of the TBDMS derivatives [6–16], the intense [M − 57] ions formed by the loss of the labile *tert.*-butyl function, together with weak [M − 15], and prominent [M − 131] and [M − 159] ions generated by the loss of CH_3, OTBDMS, and COOTBDMS from molecular ions, respectively, were useful for the structure confirmation of each amino acid.

[M − 57] ions constitute the base peaks for the amino acids having O- or COO-TBDMS derivatized side chains such as serine-2, threonine-2, aspartic acid, glutamic acid, asparagine and glutamine, and for cysteine with a S-isoBOC side chain as exemplified by the glutamine mass spectrum (Fig. 2A). For serine-1 and threonine-1, [M − 131] ions were the base peaks.

In amino acids with simple side chains such as alanine, glycine, valine, leucine, isoleucine, proline and phenylalanine the base peaks were [M − 113] ions representing the loss of $CHCH(CH_3)_2$ from [M − 57] ions as shown in the proline mass spectrum (Fig. 2B).

The base peaks of histidine and tyrosine having N-isoBOC derivatized side chains are [M − 174] ions which appear to be formed either by the loss of $N\dot{H}_2$isoBOC from [M − 57] ions or by the loss of OTBDMS and $CH(CH_3)_2$ from molecular ions as demonstrated in the mass spectrum of histidine (Fig. 2C).

For tryptophan the ion at *m/z* 130 formed by the preferential benzylic cleavage constitutes the base

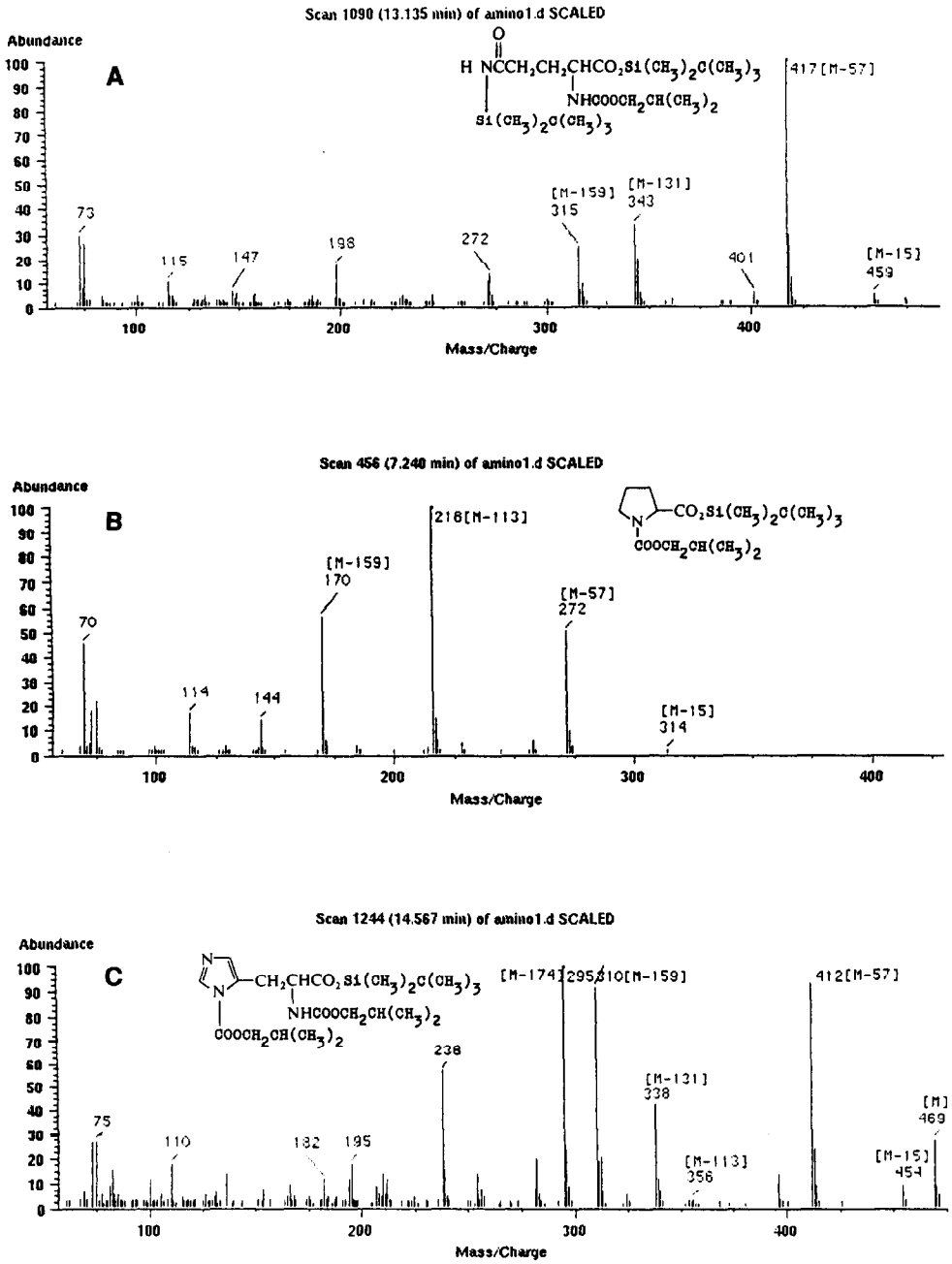

Fig. 2. Electron-impact mass spectra of N(O,S)-isoBOC TBDMS derivatives of glutamine (A), proline (B) and histidine (C).

peak while the base peaks at m/z 184 of lysine and at m/z 178 of methionine appear to represent the loss of $HCOOCH_2CH(CH_3)_2$ from [M − 174] ion, and the consecutive losses of CO and $COOCHCH(CH_3)_2$ from [M − 57] ion, respectively, as shown in Fig. 3.

CONCLUSIONS

A significant advantage of the present method is that the isobutyloxycarbonylation of the amino functions is performed in basic aqueous media for

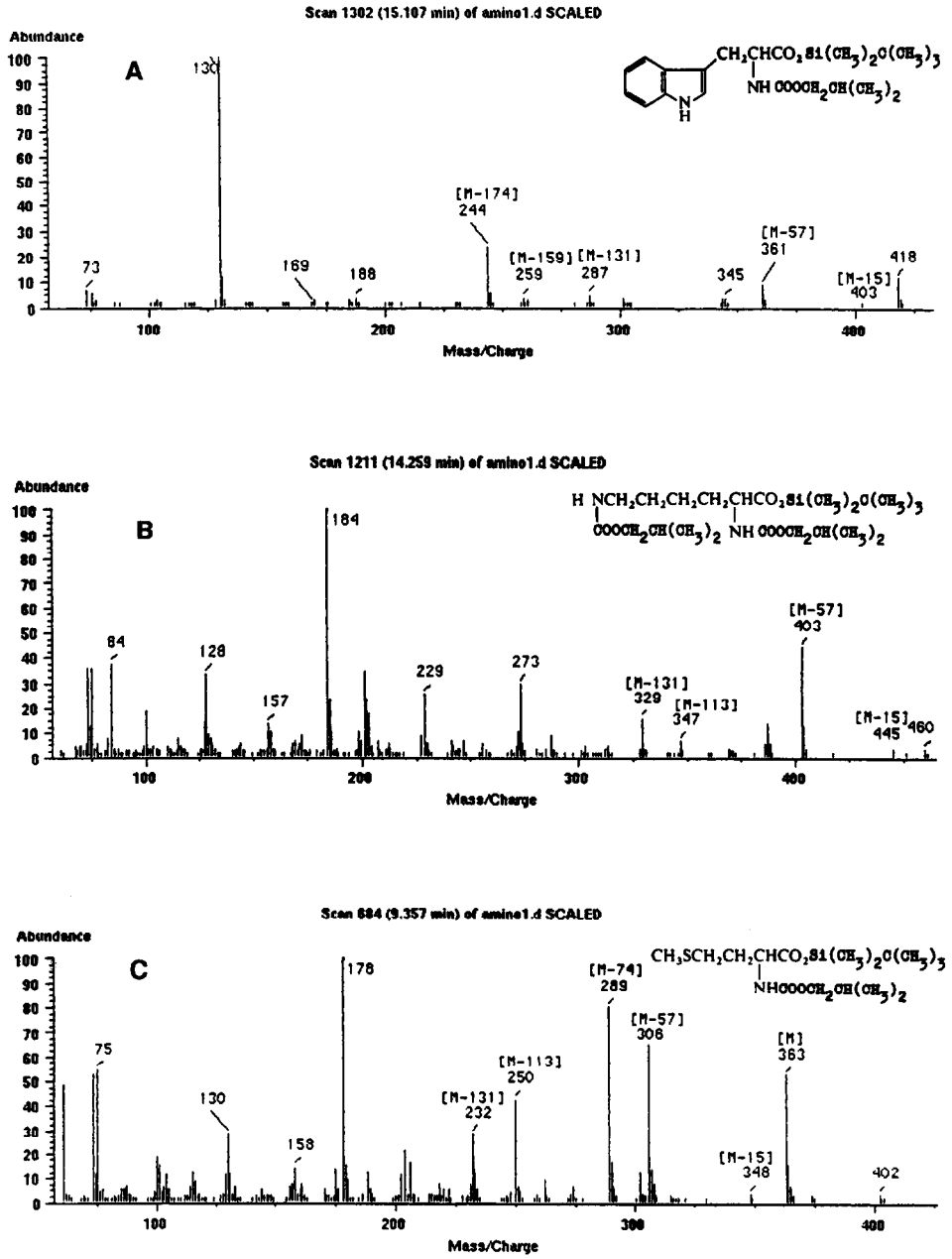

Fig. 3. Electron-impact mass spectra of N(O,S)-isoBOC TBDMS derivatives of tryptophan (A), lysine (B) and methionine (C).

the selective clean-up for zwitterionic amino acids prior to conversion to the corresponding stable TBDMS derivatives. The simple clean-up step and rapid derivatization process make this method suitable for the routine amino acid profiling works. This method was successfully applied to various food samples for the amino analysis as reported elsewhere [24]. Further investigation on the satisfactory deri-

vatization of arginine is in progress. The extension of the present method to protein and non-protein amino acid profiling analyses are under way.

ACKNOWLEDGEMENT

This work was supported by the Korea Science & Engineering Foundation (1991 project number: 911-0304-044-2).

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