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Short communication

Rapid and simultaneous analysis of protein and non-protein amino acids as N(O,S)-isobutoxycarbonyl methyl ester derivatives by capillary gas chromatography

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Abstract

A simple and rapid gas chromatographic method is described for the simultaneous determination of protein and non-protein amino acids present in aqueous samples. The amino acids were converted into their N(O,S)-isobutoxycarbonyl methyl ester derivatives and measured by gas chromatography equipped with a split inlet mode, an electronic pressure control system and a hydrogen flame ionization detector using a DB-17ht capillary column. Using this method, the 21 protein amino acids and the 33 non-protein amino acids were quantitatively and reproducibly resolved within 25 min. The calibration curves were linear in the range 0.2–20 μg of each amino acid, with correlation coefficients being above 0.998, and the relative standard deviations in each point ranged from 0.2 to 9.2%. The detection limits of amino acids were 0.09–1.15 ng per injection.

Keywords: Derivatization, GC; Amino acids; Isobutoxycarbonyl methyl esters

1. Introduction

A variety of protein and non-protein amino acids have been isolated and characterized from plant and animal sources. The simultaneous analysis of these amino acids is important because many of them have been shown to be physiologically active in the living organisms.

Several gas chromatographic (GC) methods for the simultaneous determination of protein and non-protein amino acids have been developed on the basis of the preparation of N(O,S)-heptafluorobutyryl isobutyl esters [1,2], N(O,S)-isobutoxycarbonyl (iso-BOC) *tert.*-butyldimethylsilyl (*tert.*-BDMS) derivatives [3,4], N(O)-*tert.*-BDMS derivatives [5] and ethyl chloroformate derivatives [6,7]. However, some of these methods require anhydrous conditions, high temperatures and long reaction times for the derivatization, and time-consuming GC separation. Although the methods for the ethyl chloroformate derivatives are simple and rapid, it is difficult to separate amino acids from other compounds such as amines and organic acids, which are also derivatized and detected by this method when the biological samples are analysed.

Recently, we have reported a simple and rapid method for the determination of protein amino acids [8]. This method is based on the preparation of N(O,S)-isoBOC methyl ester derivatives of amino acids by using a sonication technique and subsequent

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GC analysis using a single capillary column. This method has two main advantages. First, the amino acids in aqueous solution can be easily converted into their N(O,S)-isoBOC amino acids which are selectively extracted from the acidified reaction mixture into diethyl ether, without any step to exclude water prior to derivatization, being necessary. Second, the 22 protein amino acids could be quantitatively and reproducibly resolved as single peaks within 9 min. In this paper, we report the logical extension of this work to the rapid and simultaneous determination of a variety of protein and non-protein amino acids.

2. Experimental

2.1. Reagents

Fifty-four amino acids were examined in this study. The 21 protein amino acids: glycine (Gly), L-alanine (Ala), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-threonine (Thr), L-serine (Ser), L-proline (Pro), L-aspartic acid (Asp), L-glutamic acid (Glu), L-methionine (Met), L-hydroxyproline (Hyp), L-phenylalanine (Phe), L-lysine (Lys), L-histidine (His), L-tyrosine (Tyr), L-tryptophan (Trp) and L-cystine (Cyt) were purchased from Ajinomoto (Tokyo, Japan); and L-cysteine (Cys), L-asparagine (Asn) and L-glutamine (Gln) from Nacalai Tesque (Kyoto, Japan). The 33 non-protein amino acids: L-α-aminobutyric acid (α-ABA), α-aminoisobutyric acid (α-AIBA), DL-β-aminobutyric acid (β-ABA), DL-β-aminoisobutyric acid (β-AIBA), β-alanine (β-Ala), DL-norvaline (NVal), DL-norleucine (NLeu), γ-aminobutyric acid (GABA), DL-homoserine (HSer), L-thioproline (TPro), hippuric acid (Hip), L-ethionine (Eth), kainic acid (KNA), DL-homocysteine (HCys), L-ornithine (Orn) and DL-homocystine (HCyt) were purchased from Nacalai Tesque; ϵ-amino-*n*-caproic acid (ϵ-ACA), *o*-aminobenzoic acid (*o*-ABzA), *m*-aminobenzoic acid (*m*-ABzA), *p*-aminobenzoic acid (*p*-ABzA), and DL-α-aminopimelic acid (α-APA) from Tokyo Kasei Kogyo (Tokyo, Japan); L-*allo*-isoleucine (Alle), L-pipecolic acid (PCA), δ-amino-levulinic acid (δ-ALA), S-methyl-L-cysteine (SM-Cys), DL-α-aminoadipic acid (α-AAA), DL-2,3-diaminopropionic acid (DAPA), DL-2,4-diaminobutyric acid (DABA), L-methionine sulphone (Met-S), DL-δ-hydroxylysine (δ-HLys), DL-lanthionine (LTH) and L-cystathionine (CTH) from Sigma (St. Louis, MO, USA); and DL-γ-carboxyglutamic acid (Gla) from Calbiochem (San Diego, CA, USA). The standard stock solution (each 1 mg/ml) was prepared in 0.05 *M* hydrochloric acid and stored at 4°C. The working standard solution containing 54 amino acids (each 20 μg/ml) was made up freshly as required by mixing the stock solutions and then dilution with 0.05 *M* hydrochloric acid. 4-Piperidinecarboxylic acid as an internal standard (I.S.) was purchased from Tokyo Kasei Kogyo and was dissolved in 0.05 *M* hydrochloric acid at a concentration of 20 μg/ml. Dithioerythritol (DTE) was obtained from Nacalai Tesque and used as a 0.5 m*M* solution in distilled water. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo and used without further purification. N-Methyl-N-nitroso-*p*-toluenesulphonamide and diethyleneglycol monomethyl ether for the generation of diazomethane [9] were obtained from Nacalai Tesque. Peroxide-free diethyl ether was purchased from Dojindo Laboratories (Kumamoto, Japan). Distilled water was used fresh after purification with a Model Milli-Q Jr. water purifier (Millipore, Tokyo, Japan). All other chemicals were analytical grade.

2.2. Derivatization procedure

An aliquot of the sample containing 0.2–20 μg of each amino acid and 0.1 ml of 20 μg/ml 4-piperidinecarboxylic acid (I.S.) were pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. To this solution were added 50 μl of 0.5 m*M* DTE and 0.25 ml of 10% sodium carbonate and the total volume was made up to 1 ml with distilled water. Then 50 μl of isoBCF was added immediately and the mixture was sonicated in a Model UT-104 Ultra sonic (39 kHz) cleaner (Sharp, Tokyo, Japan) for 30 s at room temperature after shaking for a few seconds by hand. The reaction mixture was extracted with 3 ml of peroxide-free diethyl ether in order to remove the excess reagent, the ethereal extract being discarded. The aqueous layer was acidified to pH 1–2 with 2 *M* hydrochloric acid and saturated with sodium chloride, and then extracted twice with 3 ml of peroxide-free diethyl ether. The pooled ethereal

extracts were methylated by bubbling diazomethane, generated according to the microscale procedure [9], through the solution until a yellow tinge became visible. This reaction should be performed in a well ventilated hood because diazomethane is explosive and toxic. After standing for over 1 min at room temperature, the solvents were removed by evaporation to dryness at 60°C under a stream of dry air. The residue was dissolved in 0.05–0.1 ml of ethyl acetate and then 1 μl of this solution was injected into the gas chromatograph by hot-needle injection technique (needle dwell time, 3 s).

2.3. Gas chromatography

GC analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electronic pressure control (EPC) system, a split/splitless capillary inlet system and a hydrogen flame ionization detector (FID). A fused-silica capillary column of cross-linked DB-17ht (50% phenyl-50% methylpolysiloxane, J&W (Folsom, CA, USA): 20 m×0.32 mm I.D., 0.15 μm film thickness) was used. The operation conditions for the split injection system were as follows: column temperature, programmed at 4°C/min from 120 to 160°C, then programmed at 6°C/min from 160 to 200°C and at 15°C/min from 200 to 320°C; inlet helium carrier gas flow-rate, controlled at a constant 2 ml/min with EPC; split ratio, 10:1. For the splitless injection system, the column temperature was held initially at 50°C for 1 min then raised to 120°C at 70°C/min, and programmed at 4°C/min from 120 to 180°C and at 10°C/min from 180 to 310°C. The purge delay time for splitless mode was 1 min. The inlet pressure controlled with EPC, was held initially at 47 kPa for 7 min, and then programmed at 2 kPa/min from 47 to 67 kPa and at 1 kPa/min from 67 to 80 kPa. Injection and detector temperatures and 'make-up' gas flow-rate for both systems were 330°C and 30 ml/min, respectively. A chromatographic blank run (run made with no sample injected) data was subtracted from sample run data to remove baseline drift (usually caused by column bleed) using a single-column compensation function and then baseline-corrected data was recorded on the chromatogram. The peak heights of amino acids and the I.S. were measured and the peak-height ratios of amino acids against the I.S. were calculated to construct calibration curves.

3. Results and discussion

The 21 protein amino acids except for arginine and the 33 non-protein amino acids could be converted into their N(O,S)-isoBOC methyl ester derivatives by a previously reported method [8]. This method is based on the N(O,S)-isobutoxycarbonylation of amino acids with isoBCF in aqueous alkaline medium and subsequent methylation with diazomethane. In order to prevent the oxidation of sulphur amino acids during derivatization, DTE was added to the reaction mixture and peroxide-free diethyl ether was used as an extraction solvent as previously described [10]. The N(O,S)-isobutoxycarbonylation was completed within 15 s in 2.5% sodium carbonate solution with ≥10 μl of isoBCF by sonication at room temperature. Sonication technique, which can easily mix aqueous solution and oily isoBCF reagent by its vibration effect, was effective for acceleration of the reaction. The resulting N(O,S)-isoBOC amino acids could be quantitatively and selectively extracted into diethyl ether, and the subsequent methylation of the ethereal extracts could be successfully carried out by bubbling diazomethane. The derivative preparation could be performed within 10 min. The N(O,S)-isoBOC methyl ester derivatives of protein and non-protein amino acids were very stable under normal laboratory conditions.

As previously described [8], the derivatives of protein amino acids could be completely resolved as single peaks within 9 min on a capillary column of cross-linked DB-17 (15 m×0.25 mm I.D., 0.25 μm film thickness), but the separation of protein and non-protein amino acids examined in this study were incomplete on this column. Furthermore, high boiling point derivatives were eluted slowly with a broader peak width and their sensitivities were reduced because of reduction in carrier gas flow-rate at higher temperature. In order to solve these problems, we tried to introduce a EPC system and a thin-film-coated and high temperature (max. at 340°C) column DB-17ht (20 m×0.32 mm I.D., 0.15 μm film thickness). Of several GC conditions tested for this column, the three-ramp temperature pro-

grammes and EPC programmes given in Section 2 proved to give the most satisfactory separation of the 54 amino acids. The reproducibility of sample injection, expressed as percent variation from the mean peak-height ratio against the I.S., was determined from three independent injections of the standard derivative mixture. For syringe manipulation, a hot needle injection technique (the needle inserted into the injection zone was allowed to heat up for 3 s prior to the sample injection) was used to prevent the sample discrimination due to incomplete vaporization and flashback into other parts of the inlet. Table 1 shows the mean peak-height ratios of each amino acid against the I.S. and their relative standard deviations obtained from split and splitless injection analyses. A split injection system proved to be reproducible and the relative standard deviations ranged from 0.06 to 2.9%. On the other hand, a splitless injection system could introduce the most sample onto the column and had increased sensitivity, but it had reduced reproducibility because of poor refocusing. Particularly, the relative responses of high boiling point compounds such as the derivatives of LTH, CTH, Cyt and HCyt were remarkably reduced. Therefore, we adopted a hot needle injection technique in split mode as the optimum GC injection technique. As shown in Fig. 1, the 21 protein amino acids and the 33 non-protein amino acids could be separated well, as single symmetrical peaks within 25 min, on a DB-17ht capillary column under optimum GC conditions using the hot needle split injection technique, except for δ-HLys which showed two peaks due to the *allo* form present in the standard.

The calibration curves for the 54 amino acids were conducted using 4-piperidinecarboxylic acid, which showed similar behaviour to other amino acids during the derivatization and was well separated from other amino acids on a chromatogram as the I.S.. Table 2 shows linear regression data, their reproducibilities and detection limits for the 54 amino acids by the method proposed above. A linear relationship was obtained with correlation coefficients being above 0.998 in the range of 0.4-20 μg for Asn, Gln, *o*-ABzA and D-HLys and 0.2–10 μg for other amino acids. The overall reproducibility of the procedure, expressed as percent variation from the mean peak-height ratio against the I.S. in each

Table 1
Comparison of GC injection systems on the relative responses and reproducibilities for protein and non-protein amino acids

No.	Amino acid	Split injection[a]		Splitless injection[b]	
		RRF[c]	R.S.D[d] (%)	RRF	R.S.D (%)
1	α-AIBA	0.932	0.75	1.588	1.42
2	Ala	1.516	1.06	1.070	0.75
3	Gly	1.457	0.69	1.252	2.36
4	α-ABA	1.491	0.12	1.058	1.84
5	Val	1.233	0.14	0.935	1.58
6	β-Ala	1.415	0.16	0.994	1.75
7	β-ABA	1.196	0.14	1.022	1.24
8	β-AIBA	0.914	0.29	0.778	1.39
9	NVal	1.365	0.48	1.188	1.39
10	Leu	1.258	0.17	0.903	2.47
11	Alle	1.175	0.43	0.988	2.39
12	Ile	1.318	0.24	1.200	1.69
13	NLeu	1.233	0.09	1.135	2.25
14	GABA	1.043	0.11	0.826	1.60
15	Thr	0.796	0.51	0.283	1.81
16	Ser	0.573	0.53	0.373	3.76
17	Pro	1.104	0.14	1.207	2.47
18	PCA	0.951	0.74	1.071	0.79
19	HSer	0.402	1.25	0.400	1.50
20	δ-ALA	0.736	0.34	0.542	0.93
21	Asp	0.985	0.16	0.812	0.79
22	SM-Cys	0.619	0.57	0.555	0.73
23	TPro	0.926	0.22	0.877	0.91
24	Hip	0.731	0.55	0.546	2.84
25	ϵ-ACA	0.749	1.20	0.806	1.68
26	Glu	0.961	0.37	0.606	3.89
27	Met	0.484	0.32	0.374	1.74
28	*o*-ABzA	0.557	1.08	0.374	1.17
29	Hyp	0.733	0.41	0.568	1.50
30	Eth	0.403	0.76	0.298	1.66
31	α-AAA	0.955	0.89	0.710	1.20
32	Phe	1.403	0.50	1.200	1.06
33	α-APA	0.828	1.09	0.735	1.57
34	*m*-ABzA	1.159	0.22	0.968	2.70
35	Asn	0.838	0.32	0.181	1.94
36	Gla	1.004	0.40	0.897	4.81
37	*p*-ABzA	1.012	0.06	0.897	4.81
38	DAPA	1.098	0.23	0.723	0.21
39	Cys	0.894	0.17	0.670	3.73
40	KNA	1.195	2.26	1.200	1.47
41	Gln	0.589	1.36	0.270	3.15
42	DABA	1.113	0.67	0.232	5.88
43	HCys	0.533	2.35	0.452	3.07
44	Met-S	0.868	1.67	0.529	4.66
45	Orn	2.401	0.48	1.102	3.44
46	Lsy	2.337	1.31	1.197	4.50
47	His	1.400	0.75	0.684	4.65
48	Tyr	0.978	0.67	0.400	5.26
49	δ-HLys	1.454	0.58	0.562	6.79
50	Trp	1.801	1.75	0.981	4.58

Table 1. Continued

No.	Amino acid	Split injection[a]		Splitless injection[b]	
		RRF[c]	R.S.D[d] (%)	RRF	R.S.D (%)
51	LTH	0.985	0.62	0.052	5.52
52	CTH	1.003	1.95	0.187	8.70
53	Cyt	0.938	2.13	0.026	5.80
54	HCyt	0.845	2.90	0.058	4.65

[a] Split ratio: 10:1. GC conditions are given in Section 2.
[b] GC conditions are given in Section 2.
[c] Relative response factor expressed as peak-height ratio of each amino acid against the I.S.
[d] Relative standard deviation for three independent sample injections.

point, was determined from three independent analyses of the standard mixture. As shown in Table 2, the relative standard deviations for protein and non-protein amino acids were 0.2–9.2%. The minimum detectable amount of these amino acids that gave a signal-to-noise ratio of 3, under our GC conditions, was 0.09–1.15 ng injected.

4. Conclusions

These experiments have conclusively demonstrated that protein and non-protein amino acids can be

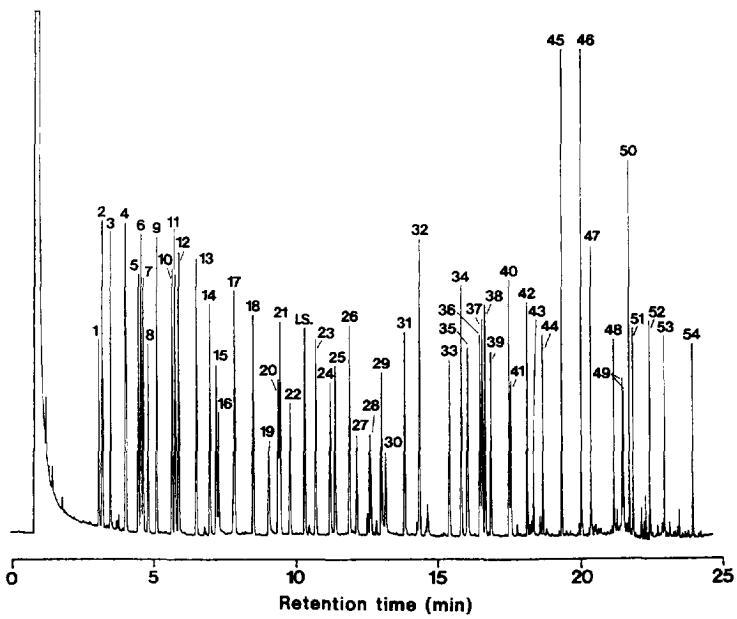

Fig. 1. Gas chromatogram obtained from the N(O,S)-isobutoxycarbonyl methyl ester derivatives of protein and non-protein amino acids (containing 4 μg of asparagine, glutamine, *o*-aminobenzoic acid and δ-hydroxylysine and 2 μg of other amino acids) separated on a DB-17ht capillary column. GC analysis is carried out by a hot needle split injection system under the conditions given in Section 2. Peaks: 1=α-aminoisobutyric acid; 2=alanine; 3=glycine; 4=α-aminobutyric acid; 5=valine; 6=β-alanine; 7=β-aminobutyric acid; 8=β-aminoisobutyric acid; 9=norvaline; 10=leucine; 11=*allo*-isoleucine; 12=isoleucine; 13=norleucine; 14=γ-aminobutyric acid; 15=threonine; 16=serine; 17=proline; 18=pipecolic acid; 19=homoserine; 20=δ-aminolevulinic acid; 21=aspartic acid; 22=S-methylcysteine; 23=thioproline; 24=hippuric acid; 25=ε-aminocaproic acid; 26=glutamic acid; 27=methionine; 28=*o*-aminobenzoic acid; 29=hydroxyproline; 30=ethionine; 31=α-aminoadipic acid; 32=phenylalanine; 33=α-aminopimelic acid; 34=*m*-aminobenzoic acid; 35=asparagine; 36=γ-carboxyglutamic acid; 37=*p*-aminobenzoic acid; 38=2,3-diaminopropionic acid; 39=cysteine; 40=kainic acid; 41=glutamine; 42=2,4-diaminobutyric acid; 43=homocysteine; 44=methionine sulphone; 45=ornithine; 46=lysine; 47=histidine; 48=tyrosine; 49=δ-hydroxylysine; 50=tryptophan; 51=lanthionine; 52=cystathionine; 53=cystine; 54=homocystine, I.S.=4-piperidinecarboxylic acid.

Table 2
Linear regression data, reproducibilities and detection limits for protein and non-protein amino acids

No.	Amino acid	Regression line[a]		Correlation coefficient[b]	R.S.D[c] (%)			Detection limit (ng)
		Slope	Intercept		0.5 μg	2 μg	10 μg	
1	α-AIBA	0.5136	−0.0379	0.9996	8.09	2.36	1.36	0.23
2	Ala	0.7553	−0.0326	0.9986	6.15	4.51	4.76	0.13
3	Gly	0.7117	−0.0247	0.9991	0.90	3.67	2.05	0.14
4	α-ABA	0.7413	0.0172	0.9995	3.71	2.43	2.95	0.11
5	Val	0.5793	0.0380	0.9996	3.68	5.01	1.28	0.15
6	β-Ala	0.9065	−0.2297	0.9987	1.08	1.54	2.49	0.12
7	β-ABA	0.6068	0.0005	0.9985	5.25	2.38	1.16	0.15
8	β-AIBA	0.4607	−0.0201	0.9993	1.73	4.08	2.95	0.22
9	NVal	0.6907	−0.0367	0.9996	5.04	2.19	2.20	0.13
10	Leu	0.5759	0.0697	0.9995	1.44	0.64	2.28	0.15
11	AIle	0.5945	−0.0175	0.9997	2.18	3.30	2.01	0.16
12	Ile	0.6300	0.0612	0.9994	2.29	1.82	1.40	0.14
13	NLeu	0.6164	−0.0045	0.9999	4.22	3.06	0.59	0.14
14	GABA	0.5111	−0.0009	0.9995	7.61	6.21	1.03	0.20
15	Thr	0.3746	0.0112	0.9997	7.38	0.19	1.70	0.60
16	Ser	0.3206	−0.0505	0.9998	1.60	5.33	0.83	0.85
17	Pro	0.5392	0.0326	0.9996	1.70	4.75	2.02	0.16
18	PCA	0.4676	0.0027	0.9998	3.24	1.79	1.10	0.18
19	HSer	0.2149	−0.0148	0.9995	3.33	3.12	1.93	0.75
20	δ-ALA	0.3441	0.0157	0.9990	6.84	2.65	2.30	0.27
21	Asp	0.4714	0.0252	0.9996	4.18	3.59	1.80	0.18
22	SM-Cys	0.3409	−0.0522	0.9997	4.17	2.61	0.28	0.33
23	TPro	0.5000	−0.0535	0.9997	4.51	2.53	1.09	0.19
24	Hip	0.3651	0.0027	0.9998	1.12	4.10	0.59	0.45
25	ε-ACA	0.3808	−0.0274	0.9990	6.89	3.61	3.17	0.26
26	Glu	0.4439	0.0471	0.9996	4.99	1.25	1.81	0.20
27	Met	0.2981	−0.0589	0.9988	2.13	2.38	3.62	0.50
28	*o*-ABzA	0.1474	−0.0074	0.9988	2.74	2.43	5.50	1.15
29	Hyp	0.3878	−0.0206	0.9990	4.12	0.48	2.77	0.60
30	Eth	0.2622	−0.0634	0.9988	2.86	4.98	0.63	0.80
31	α-AAA	0.4422	0.0256	0.9992	5.65	0.52	3.27	0.20
32	Phe	0.6933	0.0181	0.9995	1.80	2.71	2.66	0.13
33	α-APA	0.4103	0.0075	0.9994	4.87	1.85	3.33	0.23
34	*m*-ABzA	0.5846	0.0105	0.9997	6.80	3.27	1.51	0.15
35	Asn	0.1939	0.0343	0.9996	6.72	3.53	2.94	0.57
36	Gla	0.4645	0.0237	0.9993	9.17	5.18	3.21	0.20
37	*p*-ABzA	0.4873	0.0131	0.9983	2.70	5.87	2.57	0.21
38	DAPA	0.4812	0.0447	0.9989	5.80	1.07	3.58	0.20
39	Cys	0.4388	0.0235	0.9984	8.03	1.62	4.53	0.30
40	KNA	0.5282	0.0933	0.9996	8.43	1.56	0.94	0.15
41	Gln	0.1351	0.0343	0.9982	1.67	3.34	0.27	0.88
42	DABA	0.5156	0.0531	0.9995	9.01	1.97	1.61	0.21
43	HCys	0.2910	−0.0347	0.9986	4.00	0.85	2.36	0.40
44	Met-S	0.4149	0.0279	0.9988	4.44	3.23	2.91	0.23
45	Orn	1.1601	0.0640	0.9988	1.49	0.94	2.82	0.09
46	Lsy	1.1332	0.0327	0.9990	0.37	2.07	2.21	0.09
47	His	0.7171	−0.0051	0.9991	3.25	5.63	1.98	0.16
48	Tyr	0.4768	0.0293	0.9987	5.79	0.82	5.13	0.19
49	δ-HLys	0.3473	0.0261	0.9993	3.24	2.10	5.79	0.72
50	Trp	0.8911	0.0175	0.9981	6.52	4.18	5.06	0.11
51	LTH	0.4977	0.0125	0.9992	7.12	3.90	2.19	0.12
52	CTH	0.5157	−0.0481	0.9995	5.45	0.91	2.41	0.25
53	Cyt	0.4898	−0.0159	0.9990	3.45	4.80	4.11	0.29
54	HCyt	0.4425	−0.0350	0.9988	4.53	4.40	3.78	0.27

[a] Range: 0.4–20 μg for Asn, Gln, *o*-ABzA and δ-HLys; 0.2–10 μg for other amino acids.
[b] $n=18$.
[c] Relative standard deviation of peak-height ratio of each amino acid against the I.S. for three independent determinations.

accurately and precisely determined as their N(O,S)-isoBOC methyl ester derivatives by FID–GC equipped with EPC and split inlet mode, using a single capillary column. This method is simple, rapid and reproducible and the 54 amino acids can be simultaneously and quantitatively analysed within 25 min. We believe that this method provides a useful tool in biochemical research.

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