ELSEVIER

Journal of Chromatography A, 669 (1994) 125–137

JOURNAL OF CHROMATOGRAPHY A

Simultaneous gas chromatographic analysis of non-protein and protein amino acids as N(O,S)-isobutyloxycarbonyl *tert.*-butyldimethylsilyl derivatives

Chang-Hwan Oh[a], Jung-Han Kim[a], Kyoung-Rae Kim[b], Delia M. Brownson[c], Tom J. Mabry*[,c]

[a]*Department of Food Engineering, Yonsei University, Seoul 120-749, South Korea*
[b]*College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea*
[c]*Department of Botany, University of Texas at Austin, Austin, TX 78713-7640, USA*

(First received September 6th, 1993; revised manuscript received January 17th, 1994)

Abstract

A unique derivatization procedure is described for the simultaneous determination of protein and non-protein amino acids present in aqueous samples. This procedure involves N(O,S)-isobutyloxycarbonylation combined with solid-phase extraction with subsequent *tert.*-butyldimethylsilylation for gas chromatographic analysis. Using this combined procedure, linear responses were obtained in the range of 10–100 ppm, with correlation coefficients varying from 0.991 to 0.999, for the free amino acids studied except for homocysteine (0.922) and homoserine (0.982). The relative standard deviations ranged from 0.7 to 5% for most amino acids with a few exceptions. Temperature-programmed retention index (I) sets as measured on DB-5 and DB-17 dual-capillary columns were characteristic of each amino acid and thus useful for the screening of amino acids by computer I matching. The mass spectral patterns of amino acid derivatives, exhibiting characteristic $[M-57]^+$, $[M-113]^+$, $[M-131]^+$, $[M-159]^+$, $[M-174]^+$ and other ions, permitted rapid structural confirmation. The present method allowed simultaneous screening of free protein and non-protein amino acids when applied to seed samples such as almond, walnut, and sunflower seeds.

1. Introduction

We have previously reported a simple profiling method for all protein amino acids except arginine by capillary gas chromatography (GC) [1,2]. Profiling methods can simultaneously detect and identify multiple components in a single analysis and are therefore preferred for screening samples. Also, new compounds are more likely to be detected using profiling methods rather than conventional analyses that rely on identification of a few target compounds. Capillary GC is ideal for profiling analyses because of its inherent high resolving power, short analysis time, and mass spectrometry (MS) interface capabilities [3,4]. The significant advantage of our method is the unique derivatization procedure for GC analysis which includes formation of N(O,S)-isobutyloxycarbonyl (isoBOC) derivatives of free amino acids in aqueous media, recovery of the N(O,S)-isoBOC amino acids by solid-phase extraction (SPE), and the conversion

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00079-O

to the corresponding *tert.*-butyldimethylsilyl (TBDMS) derivatives. The present work was undertaken to further develop our methodology to allow simultaneous profiling of a wide variety of non-protein amino acids as well as protein amino acids, including arginine.

Within the plant kingdom, more than 600 non-protein amino acids have been identified, mostly in their free form [5]. The variety of structural types encountered within this chemical group suggest that they have diverse functions in plant metabolism, including nitrogen storage, protection of host plants from insect attack [6], and as a barrier for cross-pollination [7]. Some non-protein amino acids are known to be acutely toxic to humans and livestock [8,9]. Furthermore, recent evidence lends credence to the possibility that exogenous excitatory amino acids may play a role in chronic neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [10–12]. Our method for profiling free amino acids can be successfully applied to the rapid screening of complex mixtures as demonstrated for almond, walnut, and sunflower seed samples. This method will allow quantification of known protein and non-protein amino acids and may lead to the identification of novel non-protein amino acids. Thus, the systematic screening of plants for non-protein amino acids could indicate the potential toxicity of plants.

2. Experimental

2.1. Materials

The 51 amino acid standards examined in this study are listed in Table 1. The non-protein amino acids: α-aminoisobutyric acid, α-aminobutyric acid, β-alanine, β-aminoisobutyric acid,

Table 1
Non-protein and protein amino acids studied

Amino acid (abbreviation)	Amino acid (abbreviation)
Alanine (Ala)	D-Norleucine (Nol)
Glycine (Gly)	γ-Aminobutyric acid (GABA)
Valine (Val)	L-Pipecolic acid (Pca)
Leucine (Leu)	S-Methyl-L-cysteine (SM-Cys)
Isoleucine (Ile)	ε-Amino-*n*-caproic acid (eAca)
Proline (Pro)	DL-Ethionine (Eth)
Methionine (Met)	*o*-Aminobenzoic acid (oAbza)
Serine (Ser)	DL-Homoserine (Hser)
Threonine (Thr)	*m*-Aminobenzoic acid (mAbza)
Phenylalanine (Phe)	*p*-Aminobenzoic acid (pAbza)
Aspartic acid (Asp)	DL-Homocysteine (Hcys)
Cysteine (Cys)	DL-α-Aminoadipic acid (aAada)
Glutamic acid (Glu)	δ-Hydroxylysine (Hyl)
Asparagine (Asn)	DL-α-Aminopimelic acid (aApa)
Glutamine (Gln)	5-Hydroxy-DL-tryptophan (Hyt)
Lysine (Lys)	4-Hydroxy-L-proline (Hyp)
Histidine (His)	Ornithine (Orn)
Tryptophan (Trp)	Sarcosine (Sar)
Tyrosine (Tyr)	Cycloleucine (Cyl)
Arginine (Arg)	Pyroglutamic acid (Pgl)
α-Aminoisobutyric acid (aAiba)	2,3-diaminopropionic acid (DAPA)
α-Aminobutyric acid (aAba)	Kainic acid (Kna)
β-Alanine (bAla)	Selenomethionine (SE-Met)
β-Aminoisobutyric acid (bAiba)	Selenoethionine (SE-Eth)
L-Norvaline (Nov)	β-N-Methylamino-L-alanine (BMAA)
L-*allo*-isoleucine (A-Ile)	

L-norvaline, D-norleucine, γ-aminobutyric acid, S-methyl-L-cysteine, *o*-aminobenzoic acid, *m*-aminobenzoic acid, *p*-aminobenzoic acid, DL-homoserine, DL-homocysteine, DL-α-aminoadipic acid, δ-hydroxylysine, ornithine, sarcosine, cycloleucine, 2,3-diaminopropionic acid and kainic acid were purchased from Aldrich (Milwaukee, WI, USA); L-*allo*-isoleucine, ε-amino-*n*-caproic acid, DL-ethionine, DL-α-aminopimelic acid, 5-hydroxy-DL-tryptophan, 4-hydroxy-L-proline, selenomethionine and selenoethionine from Sigma (St. Louis, MO, USA); L-pipecolic acid from Calbiochem (San Diego, CA, USA); and β-N-methylamino-L-alanine from RBI (Natick, MA, USA). The protein amino acid standards, arginase (40–60 units/mg, from bovine liver), and isobutyl chloroformate (isoBCF) were obtained from Sigma; tetrahydrofuran (THF) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) of silylation grade from Pierce (Rockford, IL, USA). Isooctane and triethylamine of spectroanalyzed grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Diethyl ether, purchased from Oriental Chemical Industry (Seoul, South Korea), was purified as previously described [2] and stored over anhydrous sodium sulfate. Sodium chloride, purchased from Junsei (Tokyo, Japan), was washed successively with methanol, acetone, dichloromethane and diethyl ether, followed by drying under vacuum (100°C, 1 h). Sodium carbonate and sulfuric acid were obtained from Duksan (Seoul, South Korea), and *n*-hydrocarbon standards (C_{12}–C_{36}, even numbers only) from Polyscience (Niles, IL, USA). Chromosorb P (acid washed, 80–100 mesh) was obtained from Supelco (Bellefonte, PA, USA). A U-shaped glass column (6 mm I.D.), packed with Chromosorb P (2.3 g), was washed successively with methanol, dichloromethane and diethyl ether. The Chromosorb P column was activated under vacuum (150°C, 2 h) prior to being used as a solid-phase extraction column. Seed material including walnuts, almonds, and sunflower seeds were obtained from a local supermarket (Seoul, South Korea). All samples and standards were stored desiccated at −20°C until analyzed.

2.2. Amino acid and internal standard solutions

Four separate amino acid working solutions were prepared at a concentration of 0.5 μg of each amino acid per μl of 0.1 *M* HCl. One working solution contained all protein amino acids except for arginine, the second contained all protein amino acids, and the third contained only arginine. The fourth working solution contained 30 non-protein amino acid standards. Two separate internal standard (I.S.) solutions were prepared by dissolving norvaline and 3,4-dimethoxybenzoic acid at a concentration of 5 μg per μl of 0.1 *M* HCl and methanol, respectively.

2.3. N(O,S)-Isobutyloxycarbonylation of amino acids

Distilled water (1.2 ml) containing 20 μg of norvaline as I.S. was added to a 100-μl aliquot of each amino acid solution. After adjusting the pH to 11 with a 5% sodium carbonate solution (0.2–0.4 ml), 0.5 ml of isoBCF were added, and the mixture was vortexed for 5 min at room temperature. Next, the solution was washed with diethyl ether (3 × 2 ml) to remove excess isoBCF. The resulting N(O,S)-isobutyloxycarbonyl (isoBOC) amino acids were subsequently solid-phase extracted and *tert.*-butyldimethylsilylated as described below. The effect of basicity on the N(O,S)-isobutyloxycarbonylation using protein amino acids and 3,4-dimethoxybenzoic acid as I.S. was evaluated at three different pH values (pH 11, 12 and 13).

2.4. Solid-phase extraction

The aqueous solution was acidified (pH 1–2) with 10% sulfuric acid, and saturated with sodium chloride. The solution was then loaded onto the Chromosorb P column for the SPE as described previously [13]. Briefly, by applying nitrogen pressure, the aqueous phase was allowed to advance until 80% of the column was moist. Next, the N(O,S)-isoBOC amino acids were eluted with diethyl ether from the

Chromosorb P and the eluate was subjected to *tert.*-butyldimethylsilylation as described below.

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

A small fraction (about 100 μl) of each eluate containing N(O,S)-isoBOC amino acids was evaporated to dryness under a gentle stream of nitrogen (50°C). To the residue, 20 μl of THF and 20 μl of MTBSTFA were added, and the solution was heated at 60°C for 20 min to form TBDMS derivatives. The reaction solution was then directly analyzed by GC and GC–MS.

Precision of the overall procedures was evaluated using three different types of solutions: the protein amino acid mixture without arginine, the non-protein amino acid mixture, and the arginine solution. These solutions were prepared with 10, 20, 50 or 100 μg of each amino acid and 20 μg of norvaline as the internal standard. In place of THF, acetonitrile and isooctane containing triethylamine were investigated as solvents for TBDMS derivatization.

2.6. Conversion of arginine into ornithine

An aliquot (1.5 ml) of a solution containing 50 μg of both arginine and the internal standard was adjusted to pH 9.4–9.6 with 5% sodium carbonate solution. The activated arginase solution (0.1 ml) was added. The mixture was incubated at 37°C for 20 min and centrifuged to discard the precipitate [14,15]. Then, the solution was subjected to the isoBOC reaction, SPE and TBDMS derivatization as described above. Similarly, the effect of arginase treatment on the overall procedure of protein amino acids other than arginine was examined.

2.7. Sample preparation

A 500-mg sample of seed material (almond, walnut or sunflower) was finely ground with a mortar and pestle. The material was vortexed with distilled water (4 × 1 ml) and centrifuged (350 *g*, 5 min). The supernatant (1 ml) was treated with a saturated picric acid solution (200 μl), gently shaken, and then centrifuged (2000 rpm, 2 min). After discarding the precipitate, a few drops of 10% sulfuric acid were added to the solution, followed by sequential washing with ethyl acetate (3 × 2 ml) and diethyl ether (2 × 2 ml). The aqueous layer was basified to pH 11 and then subjected to the isoBOC reaction, SPE and TBDMS derivatization as described above.

2.8. Gas chromatography

The GC analysis was conducted with a Hewlett-Packard HP 5890A gas chromatograph, equipped with a split/splitless and on-column capillary inlet system, two flame ionization detectors, and an HP 3392A integrator, and interfaced to an HP 5895A GC Chemstation (Hewlett-Packard, Avondale, PA, USA). For optimization of the procedure, and precision tests, samples (*ca.* 0.2-μl aliquots) were injected in the on-column mode and chromatographed on a fused-silica Ultra-1 capillary column (25 m × 0.32 mm I.D., 0.17 μm film thickness; Hewlett-Packard). Nitrogen, at a flow-rate of 21 cm/s, was used as the carrier gas. After an initial hold time of 1 min at 80°C, the oven temperature was raised to 150°C at a rate of 30°C/min. After holding for 1 min at 150°C, the oven temperature was then programmed to 280°C at a rate of 3°C/min. The derivative yields were evaluated based on the comparison of either peak area of each amino acid derivative or peak area ratio to the internal standard.

For the retention index (*I*) measurements and profiling of amino acids, samples (*ca.* 1-μl aliquots) were injected in the split mode (30:1). The split injector was connected to deactivated fused-silica tubing (1 m × 0.25 mm I.D.) and a Chromfit "Y" splitter (Unimetrics, Shorewood, IL, USA) that allowed the attachment of both DB-5 and DB-17 fused-silica capillary columns (30 m × 0.25 mm I.D. and 0.241 μm film thickness, J & W Scientific, Folsom, CA, USA). The oven temperature was held initially at 150°C for 2 min and then programmed to 280°C at a rate of 3°C/min. The split/splitless injector and detector temperatures were 280 and 300°C, respectively. The two flame ionization detection (FID) signals

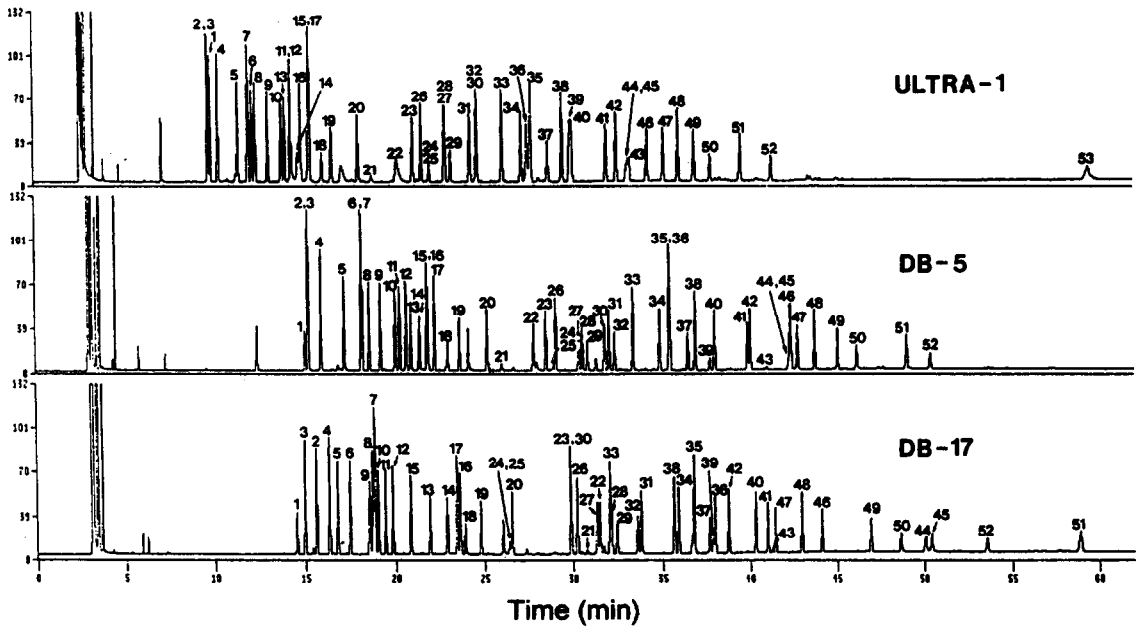

Fig. 1. Chromatograms of the 51 non-protein and protein amino acid mixture as their N(O,S)-isoBOC TBDMS derivatives separated on Ultra-1 (25 m × 0.32 mm I.D.), DB-5 (30 m × 0.25 mm I.D.) and DB-17 (30 m × 0.25 mm I.D.) dual fused-silica capillary columns. GC conditions are in the text. Peaks: 1 = α-aminoisobutyric acid; 2 = sarcosine; 3 = alanine; 4 = glycine; 5 = α-aminobutyric acid; 6 = valine; 7 = β-alanine; 8 = β-aminoisobutyric acid; 9 = norvaline; 10 = leucine; 11 = *allo*-isoleucine; 12 = isoleucine; 13 = threonine-1; 14 = serine-1; 15 = D-norleucine; 16 = proline; 17 = γ-aminobutyric acid; 18 = cycloleucine; 19 = pipecolic acid; 20 = S-methylcysteine; 21 = pyroglutamic acid; 22 = 4-hydroxyproline; 23 = methionine; 24 = serine-2; 25 = threonine-2; 26 = ε-aminocaproic acid; 27 = ethionine; 28 = selenomethionine; 29 = *o*-aminobenzoic acid; 30 = DL-homoserine; 31 = phenylalanine; 32 = selenoethionine; 33 = aspartic acid; 34 = β-N-methylamino-L-alanine; 35 = 2,3-diaminopropionic acid; 36 = *m*-aminobenzoic acid; 37 = cysteine; 38 = glutamic acid; 39 = asparagine; 40 = *p*-aminobenzoic acid; 41 = homocysteine; 42 = α-aminoadipic acid; 43 = glutamine; 44 = δ-hydroxylysine-1; 45 = δ-hydroxylysine-2; 46 = ornithine; 47 = α-aminopimelic acid; 48 = kainic acid; 49 = lysine; 50 = histidine; 51 = tryptophan; 52 = tyrosine; 53 = 5-hydroxy-DL-tryptophan (peak numbers correspond to Table 2).

were processed simultaneously in dual channel mode by the GC Chemstation [16]. For the *I* measurements, a standard solution of *n*-hydrocarbons (C_{12}–C_{36}, even numbers only) in isooctane was co-injected with the samples.

All the GC analyses were performed in triplicate.

2.9. Gas chromatography–mass spectrometry

An HP 5890A Series II gas chromatograph, interfaced to an HP 5970B mass-selective detector (70 eV, electron impact mode) and on-line to an HP 59940A MS Chemstation system, was used with an HP-5 cross-linked capillary column (25 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 at 200°C for 2 min then raised to 280°C at a rate of 5°C/min. The interface temperature was 300°C. The mass range scanned was 60–650 a.m.u. at a rate of 1.0 scan/s.

3. Results and discussion

3.1. GC and GC–MS analysis

Following the isoBOC reaction and SPE with subsequent TBDMS derivatization, each of the non-protein amino acids studied was converted

Table 2
Gas chromatographic and mass spectral data of N(O,S)-isoBOC TBDMS derivatives of non-protein and protein amino acids

No.	Amino acid	GC I^a data set		M_r	Mass spectral data set[b]						
		DB-5	DB-17		[M − 57]	[M − 15]	[M − 113]	[M − 131]	[M − 159]	[M − 174]	Other ions
1	aAiba	1695.7	1840.7	317	260 (53)	302 (2)	204 (100)	186 (15)	158 (95)	143 (41)	
2	Sar	1701.4	1878.8	303	246 (41)	288 (0)	190 (100)	172 (0)	144 (6)	129 (0)	
3	Ala	1702.7	1856.8	303	246 (33)	288 (1)	190 (100)	172 (2)	144 (23)	129 (2)	
4	Gly	1725.9	1903.8	289	232 (26)	274 (0)	176 (100)	158 (8)	130 (4)	115 (8)	
5	aAba	1767.8	1919.4	317	260 (54)	302 (1)	204 (100)	186 (2)	158 (37)	143 (3)	
6	Val	1800.0	1942.9	331	274 (35)	316 (1)	218 (100)	200 (2)	172 (28)	157 (3)	
7	bAla	1800.0	1992.4	303	246 (30)	288 (1)	190 (100)	172 (14)	144 (4)	129 (32)	
8	bAiba	1813.8	1988.3	317	260 (57)	302 (1)	204 (100)	186 (8)	158 (2)	143 (30)	
9	Nov	1833.7	1981.4	331	274 (50)	316 (1)	218 (100)	200 (7)	172 (26)	157 (9)	
10	Leu	1860.1	2000.0	345	288 (38)	330 (1)	232 (100)	214 (2)	186 (36)	171 (3)	
11	A-Ile	1867.9	2012.8	345	288 (49)	330 (1)	232 (100)	214 (6)	186 (28)	171 (4)	
12	Ile	1880.2	2027.3	345	288 (39)	330 (1)	232 (100)	214 (2)	186 (35)	171 (3)	
13	Thr-1	1887.6	2098.3	333	276 (11)	318 (1)	220 (64)	202 (100)	174 (9)	159 (4)	
14	Ser-1	1903.1	2131.8	319	262 (12)	304 (1)	206 (46)	188 (100)	160 (15)	145 (5)	
15	Nol	1917.7	2061.5	345	288 (58)	330 (1)	232 (100)	214 (2)	186 (41)	171 (5)	
16	Pro	1918.1	2157.1	329	272 (50)	314 (1)	216 (100)	198 (0)	170 (56)	155 (0)	
17	GABA	1930.5	2150.4	317	260 (31)	302 (1)	204 (35)	186 (8)	158 (2)	143 (16)	160 (100)
18	Cyl	1955.1	2167.2	343	286 (52)	328 (2)	230 (79)	212 (4)	184 (100)	169 (20)	
19	Pca	1975.9	2196.6	343	286 (64)	328 (2)	230 (54)	212 (1)	184 (100)	169 (1)	
20	SM-Cys	2026.5	2259.1	349	292 (28)	334 (1)	236 (7)	218 (6)	190 (7)	175 (100)	
21	Pgl	2053.0	2408.3	343	286 (40)	328 (1)	230 (29)	212 (0)	184 (3)	169 (0)	186 (100)
22	Hyp	2112.0	2434.9	345	288 (100)	330 (3)	232 (94)	214 (0)	186 (85)	171 (0)	
23	Met	2135.0	2376.3	363	306 (65)	348 (3)	250 (40)	232 (28)	204 (21)	189 (5)	178 (100)
24	Ser-2	2147.9	2254.4	433	376 (100)	418 (4)	320 (14)	302 (33)	274 (11)	259 (4)	
25	Thr-2	2147.3	2256.9	447	390 (100)	432 (4)	334 (7)	316 (28)	288 (17)	273 (5)	
26	eAca	2152.6	2387.8	345	288 (25)	330 (1)	232 (9)	214 (22)	186 (3)	171 (29)	188 (100)
27	Eth	2194.4	2428.6	377	320 (68)	362 (2)	264 (28)	246 (15)	218 (13)	203 (3)	75 (100)
28	SE-Met	2200.0	2458.5	411	354 (75)	396 (1)	298 (57)	280 (16)	252 (20)	237 (2)	226 (100)
29	oAbza	2211.2	2470.8	351	294 (48)	336 (1)	238 (100)	220 (26)	192 (0)	177 (2)	
30	Hser	2243.8	2375.1	447	390 (100)	432 (2)	334 (21)	316 (15)	288 (26)	273 (0)	
31	Phe	2253.5	2521.7	379	322 (82)	364 (2)	266 (100)	248 (14)	220 (18)	205 (57)	
32	SE-Eth	2264.2	2514.2	425	368 (83)	410 (2)	312 (51)	294 (18)	266 (23)	251 (1)	240 (100)
33	Asp	2300.1	2456.9	461	404 (100)	446 (2)	348 (3)	330 (12)	302 (15)	287 (49)	
34	BMAA	2351.0	2600.0	432	375 (20)	417 (2)	319 (3)	301 (2)	273 (1)	258 (29)	144 (100)
35	DAPA	2369.4	2634.8	418	361 (36)	403 (2)	305 (9)	287 (2)	259 (3)	244 (26)	289 (100)
36	mAbza	2369.6	2682.2	351	294 (100)	336 (4)	238 (40)	220 (37)	192 (0)	174 (0)	
37	Cys	2407.0	2670.2	435	378 (100)	420 (3)	322 (23)	304 (6)	276 (16)	261 (25)	
38	Glu	2422.9	2590.7	475	418 (100)	460 (3)	362 (2)	344 (89)	316 (58)	301 (2)	
39	Asn	2453.3	2678.3	460	403 (100)	445 (5)	347 (4)	329 (19)	301 (3)	286 (27)	
40	pAbza	2462.8	2775.2	351	294 (100)	336 (3)	238 (5)	220 (56)	192 (0)	174 (0)	
41	Hcys	2531.8	2802.2	449	392 (83)	434 (2)	336 (22)	218 (19)	190 (11)	172 (1)	156 (100)
42	aAada	2537.7	2713.4	489	432 (97)	474 (2)	376 (6)	358 (87)	330 (100)	315 (3)	
43	Gln	2573.6	2814.3	474	417 (100)	459 (4)	361 (2)	343 (33)	315 (24)	300 (2)	
44	Hyl-1	2618.8	3167.5	590	533 (62)	575 (2)	477 (0)	459 (18)	431 (0)	416 (2)	328 (100)
45	Hyl-2	2623.2	3180.8	590	533 (58)	575 (2)	477 (0)	459 (8)	431 (0)	416 (3)	328 (100)
46	Orn	2623.9	2938.0	446	389 (43)	431 (2)	333 (8)	315 (9)	287 (0)	272 (0)	170 (100)

Table 2 (*continued*)

No.	Amino acid	GC I^a data set		M_r	Mass spectral data set[b]						
		DB-5	DB-17		[M − 57]	[M − 15]	[M − 113]	[M − 131]	[M − 159]	[M − 174]	Other ions
47	aApa	2640.9	2822.8	503	446 (98)	488 (2)	390 (8)	372 (59)	344 (100)	329 (11)	
48	Kna	2679.8	2887.2	541	484 (77)	526 (3)	428 (3)	410 (2)	382 (100)	367 (1)	
49	Lys	2732.2	3050.4	460	403 (44)	445 (3)	347 (6)	329 (15)	301 (2)	286 (0)	184 (100)
50	His	2775.1	3115.1	469	412 (93)	454 (9)	356 (3)	338 (42)	310 (91)	295 (100)	
51	Trp	2870.3	3413.8	418	361 (8)	403 (1)	305 (1)	287 (4)	259 (3)	244 (23)	130 (100)
52	Tyr	2913.8	3274.0	495	438 (12)	480 (1)	382 (9)	364 (6)	336 (3)	321 (100)	
53	Hyt	n.d.[c]	n.d.[c]	534	477 (5)	519 (0)	421 (3)	403 (0)	375 (4)	360 (16)	246 (100)

[a] Retention index (I) values on DB-5 and DB-17 (30 m × 0.25 mm I.D., 0.25 μm film thickness) capillary columns programmed from 150°C (held for 2 min) to 280°C at 3°C /min. Relative standard deviations ranged from 0.01 to 0.05% for three measurements.

[b] m/z Values, with relative abundances of ions (%) in parentheses.

[c] Not detected.

to a single derivative similar to most protein amino acids [1,2]. The separation of 53 amino acid derivatives on three different fused-silica capillary columns is shown in Fig. 1. Each amino acid displayed a single symmetrical peak, except for serine and threonine both of which exhibited two peaks as previously reported [1,2]. 5-Hydroxy-DL-tryptophan, the last eluting peak on the Ultra-1 column, was not eluted from the DB-5 and DB-17 columns. Two peaks, labeled as Hyl-1 and Hyl-2, detected for δ-hydroxy-DL-lysine were due to the *allo* form present in the standard.

The Ultra-1 and DB-5 columns of low polarity resolved the majority of the peaks for the 53 amino acid derivatives. However, the Ultra-1 column did not resolve 17 peaks and the DB-5 column did not resolve 13 peaks. The DB-17 column of medium polarity gave a better resolution with only 8 peaks not separated. The peaks which were unresolved on the DB-5 column were well separated on DB-17, and *vice versa*. Therefore, the DB-5 and DB-17 dual-capillary column system provided a complete separation. Moreover, the elution order of most amino acids on the two columns was very different. The temperature-programmed I sets, determined on dual-capillary column system, were characteristic (I sets for all peaks are given in Table 2) and useful as a cross-check for each amino acid. The computer I library matching provided rapid screening analysis as described elsewhere [16].

The amino acid derivatives were subjected to GC–MS analysis and the electron-impact MS data are summarized in Table 2. Similar to the protein amino acids [2], the base peaks of most non-protein amino acid derivatives were either $[M-57]^+$ or $[M-113]^+$ ions. Also, $[M-131]^+$, $[M-159]^+$ and $[M-174]^+$ ions were useful for the structural confirmation of non-protein amino acid derivatives.

For acidic non-protein amino acids such as α-aminoadipic acid, α-aminopimelic acid and kainic acid, and cyclic amino acids like cycloleucine and pipecolic acid, the base peaks were $[M-159]^+$ ions as shown in the representative mass spectrum for kainic acid (Fig. 2A). The base peaks of γ-aminobutyric acid, ε-aminocaproic acid and pyroglutamic acid were $[M-157]^+$ ions, possibly formed by the loss of CO_2 from $[M-113]^+$ ions as demonstrated in the mass spectrum of γ-aminobutyric acid (Fig. 2B). Similar to methionine [2], $[M-185]^+$ ions constituted the base peaks for selenomethionine and selenoethionine (Fig. 2C), whereas the ion at m/z 75 representing $CH_2SCH_2CH_3$ was the base peak for ethionine (Fig. 3A). Similar to

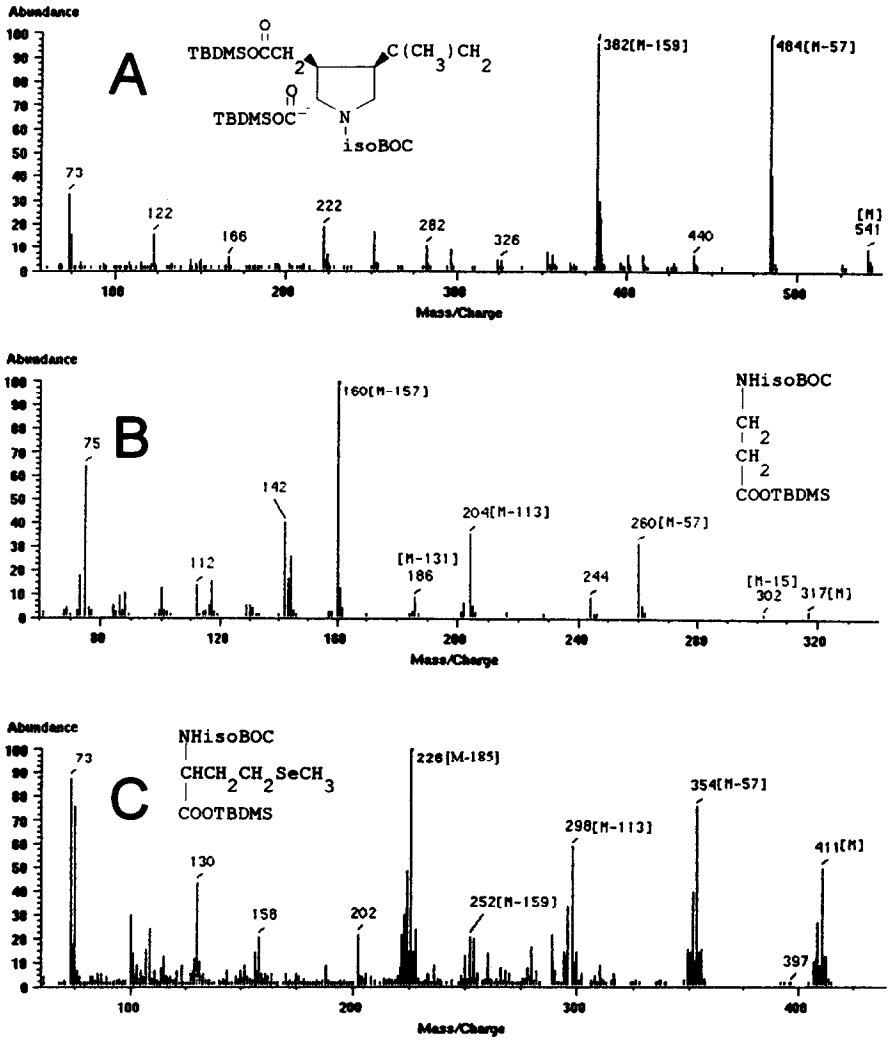

Fig. 2. Electron-impact mass spectra of N(O,S)-isoBOC TBDMS derivatives of (A) kainic acid, (B) γ-aminobutyric acid and (C) selenomethionine.

methionine, the base peak for ornithine was m/z 170, which appeared to represent either the loss of $HCOOCH_2CH(CH_3)_2$ from the $[M-174]^+$ ion or the loss of H_2N-isoBOC from $[M-159]^+$ ion. The ion at m/z 156 of homocysteine was probably formed by the loss of HS-isoBOC from $[M-159]^+$ ion (Fig. 3B). For diaminopropionic acid, the loss of CHNH-isoBOC resulted in a base peak at m/z 289 (Fig. 3C). The base peak at m/z 328 for δ-hydroxylysine was most likely formed by the losses of HO-TBDMS and CH_2NH-isoBOC from the molecular ion.

3.2. Combined N(O,S)-isoBOC reaction, SPE and TBDMS derivatization

The isoBOC reaction requires a basic pH condition. In 2.5% sodium carbonate solution, protein amino acids, except arginine, were easily

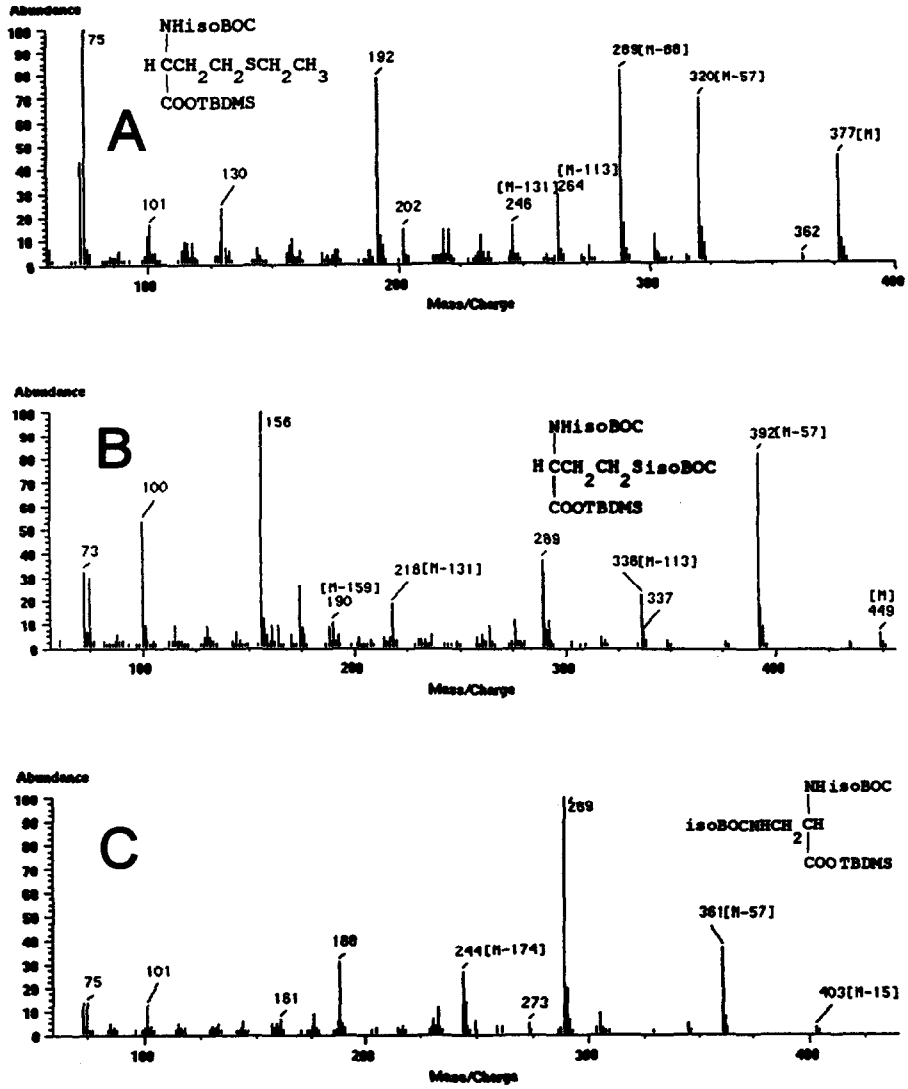

Fig. 3. Electron-impact mass spectra of N(O,S)-isoBOC TBDMS derivatives of (A) ethionine, (B) DL-homocysteine and (C) 2,3-diaminopropionic acid.

converted to their N(O,S)-isoBOC derivatives [1,2,15]. However, systematic variation of pH demonstrated that extreme basicity was detrimental to the yields of N(O,S)-isoBOC amino acid derivatives, as shown for 18 protein amino acids in Fig. 4. As the pH was raised from 11 to 12, slow increases in yields of derivatives for most amino acids were obtained; however, there were abrupt reductions in yields for derivatives of serine, asparagine and glutamine. When reacted at pH 13, the yields of derivatives were significantly reduced for most amino acids with a few exceptions. The extremely low yield obtained for tyrosine at pH 13 was probably due to the poor reactivity of its phenoxide ion with isoBCF. Therefore, pH 11 was selected for the

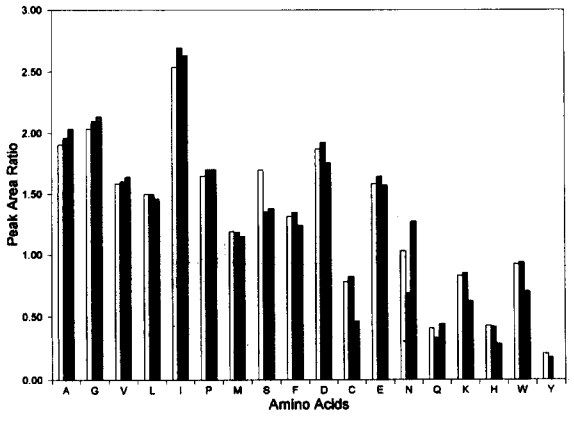

Fig. 4. The effect of basicity on isobutyloxycarbonylation of protein amino acids: A = alanine; G = glycine; V = valine; L = leucine, I = isoleucine, P = proline; M = methionine; S = serine; F = phenylalanine; D = aspartic acid; C = cysteine; E = glutamic acid; N = asparagine; Q = glutamine; K = lysine; H = histidine; W = tryptophan; Y = tyrosine. The peak area ratio of each amino acid to the internal standard is plotted against pH 11 (open bars), pH 12 (solid bars) and pH 13 (hatched bars).

simultaneous isoBOC reaction of protein and non-protein amino acids.

Prior to the isoBOC reaction, arginine requires conversion to ornithine with arginase as noted by Makita *et al.* [15]. However, arginase treatment generally gave reduced yields for most amino acids. Therefore, the linear response for arginine was determined separately.

After removal of excess isoBCF with subsequent acidification, the N(O,S)-isoBOC amino acids were recovered from the aqueous phase by SPE using hydrophilic Chromosorb P as the solid sorbent. Because of inherent advantages of SPE [13,17,18] compared with laborious solvent extraction [2,15], the SPE method permitted an efficient and rapid extraction of the N(O,S)-isoBOC amino acids.

As reported previously [2], the TBDMS derivatization of most N(O,S)-isoBOC protein amino acids in acetonitrile was favorable. However, persilylation of alanine and glycine was found to occur frequently. Therefore, less polar solvents such as THF or isooctane containing triethylamine were tested. In both solvents, persilylation was significantly reduced; unfortunately, TBDMS derivatization of most amino acids was also diminished in the latter solvent. For most protein amino acids, the overall TBDMS-derivative yields and stability were satisfactory when reacted in THF at 60°C for 20 min. Therefore, these conditions were selected for the simultaneous TBDMS derivatization of the N(O,S)-isoBOC protein and non-protein amino acids.

The combined method of N(O,S)-isoBOC reaction, SPE and TBDMS derivatization of all amino acids, under the selected conditions, were examined to measure the overall precision and accuracy. Peak area measurements to test the

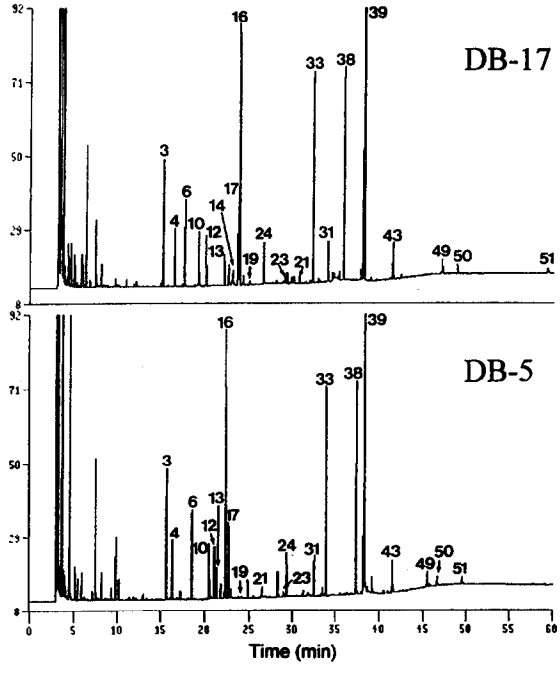

Fig. 5. Chromatograms of non-protein and protein amino acids in *Prunus dulcis* (almond) separated on DB-17 (30 m × 0.25 mm I.D.) and DB-5 (30 m × 0.25 mm I.D.) dual capillary column system. GC conditions are in the text. Peak numbers correspond to those in Fig. 1.

linear relation between responses and amounts were performed using on-column injection mode, a method of superior precision. As listed in Table 3, linear responses were obtained for most amino acids in the range of 10-100 ppm with correlation coefficients varying from 0.991–0.999, except for DL-homoserine (0.983) and DL-homocysteine (0.923) which are inherently unstable in aqueous solution. The relative standard deviations ranged from 0.7 to 5.0% with the exception of DL-homoserine (5.7–7.6), DL-homocysteine (7.2–12.0), histidine (4.5–8.6) and 5-hydroxy-DL-tryptophan (3.0–6.7). The overall reproducibility and linearity of the three combined steps for derivatization of amino acid standards appear to be sufficient for their quantitative measurements in unknown samples.

3.3. Screening of amino acids

The usefulness of the present method for free amino acid profiling and screening of seed samples is established (Figs. 5–7). The combined procedures demonstrate a highly specific extraction to obtain most free amino acids from aqueous samples. Simultaneous screening with accurate confirmation of protein and non-protein amino acids present in the seeds was achieved.

The non-protein amino acids: L-pipecolic acid and pyroglutamic acid were detected in almond (Fig. 5). In walnut, α-aminoisobutyric acid, pyroglutamic acid and ornithine were found (Fig. 6), while L-*allo*-isoleucine was detected in sunflower seed (Fig. 7). In all three samples, γ-aminobutyric acid, which is known to be an

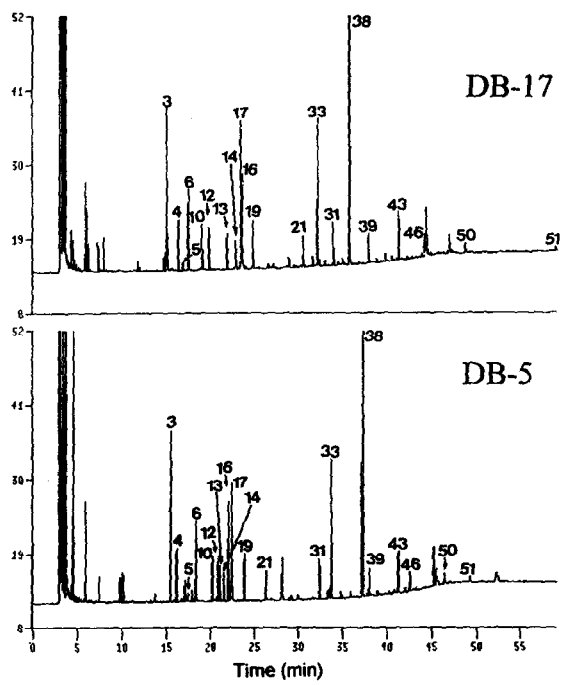

Fig. 6. Chromatograms of non-protein and protein amino acids in *Juglands regia* (walnut) separated on DB-17 (30 m × 0.25 mm I.D.) and DB-5 (30 m × 0.25 mm I.D.) dual capillary column system. GC conditions are in the text. Peak numbers correspond to those in Fig. 1.

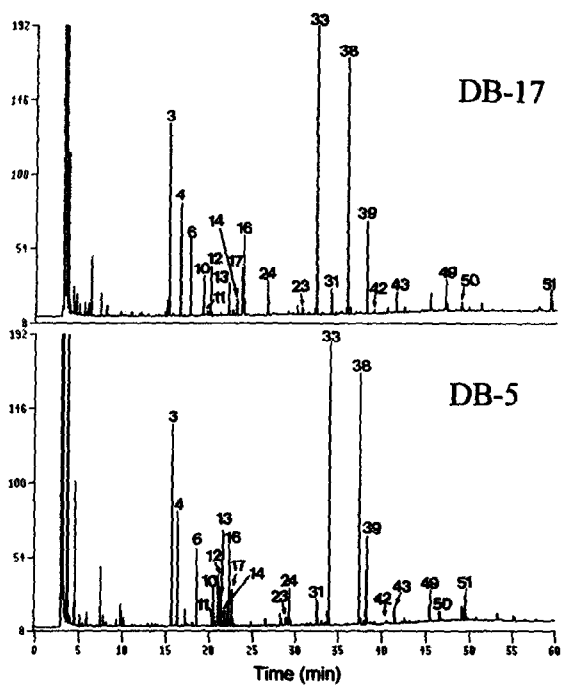

Fig. 7. Chromatograms of non-protein and protein amino acids in *Helianthus annuus* (sunflower seed) separated on DB-17 (30 m × 0.25 mm I.D.) and DB-5 (30 m × 0.25 mm I.D.) dual capillary column system. GC conditions are in the text. Peak numbers correspond to those in Fig. 1.

Table 3
Linear regression analysis for the calibration graphs of non-protein and protein amino acids as their N(O,S)-isoBOC TBDMS derivatives

Amino acid	Regression line[a]		Correlation coefficient,	Amino acid	Regression line[a]		Correlation coefficient,
	m	*b*	*r*		*m*	*b*	*r*
aAiba	0.902	0.283	0.999	Hser	0.286	−0.127	0.982
Sar	0.858	0.141	0.998	Phe	0.887	0.066	0.999
Ala	1.015	0.137	0.999	SE-Eth	0.508	0.044	0.999
Gly	1.136	0.111	0.999	Asp	1.105	0.190	0.999
aAba	0.992	0.135	0.999	BMAA	0.776	0.075	0.998
Val	0.876	0.106	0.999	DAPA	1.109	0.265	0.999
bAla	1.563	0.457	0.998	mAbza	0.726	0.084	0.999
bAiba	0.949	0.341	0.991	Cys	0.701	−0.121	0.998
Leu	0.944	−0.188	0.995	Glu	1.012	0.103	0.999
A-Ile	0.921	0.188	0.996	Asn	0.661	−0.211	0.998
Ile	0.951	0.029	0.999	pAbza	1.048	0.140	0.998
Thr	0.838	0.200	0.994	Hcys	1.031	−1.052	0.922
Ser	0.799	0.068	0.999	aAada	0.957	0.154	0.999
Nol	0.906	0.075	0.999	Gln	0.528	−0.071	0.999
Pro	0.941	0.105	0.999	Hyl	0.391	0.052	0.999
GABA	1.171	0.312	0.998	Orn	0.668	0.079	0.999
Cyl	0.799	−0.098	0.999	Arg	0.417	0.041	0.998
Pca	0.644	0.135	0.996	aApa	0.726	0.110	0.998
SM-Cys	0.801	0.173	0.994	Kna	0.456	0.205	0.999
Hyp	0.718	0.129	0.999	Lys	0.639	0.053	0.999
Met	0.849	−0.156	0.999	His	0.278	0.060	0.994
eAca	1.090	0.219	0.997	Trp	0.617	0.006	0.999
Eth	0.772	0.079	0.997	Tyr	0.604	−0.217	0.998
SE-Met	0.526	0.019	0.999	Hyt	0.871	−0.137	0.998
oAbza	0.713	−0.064	0.998				

[a] m = Slope = relative mass response = mean peak area ratio of amino acid × mass of I.S./mass of amino acid; b = y-intercept.

inhibitory transmitter in the central nervous system [19], was detected.

4. Conclusions

The method of GC analysis of amino acids described here has three main advantages. First, the procedure of isobutyloxycarbonylation and SPE prior to conversion to their corresponding TBDMS derivatives allows efficient, rapid and highly specific extraction of most free amino acids from aqueous samples. Second, under the optimized conditions employed here, the overall reproducibility of the combined procedure is satisfactory for precise and accurate quantification of amino acids. Dual columns, with differing polarity, provide complete separation of amino acids with characteristic I sets that can be used for routine amino acid screening. Moreover, since the mass spectra of amino acids as N(O,S)-isoBOC TBDMS derivatives are also characteristic, GC–MS ensures structural confirmation and positive identification. As exemplified by the three seed samples, a dual-capillary column GC system in conjunction with computer I matching can be successfully utilized to obtain simultaneous profiles of non-protein and protein amino acids in a variety of aqueous extracts. An extension of this new method for the rapid profiling

and screening of various plant samples for free amino acids is in progress.

5. Acknowledgement

This work was supported in part by the Korean Science and Engineering Foundation (1991 project number 911-0304-044-2) and in part by the National Institutes of Health (AG-10637), the Robert A. Welch Foundation (Grant F-130) and NATO (Grant CRG 910429).

6. References

[1] K.-R. Kim, J.-H. Kim and C.-H. Oh, *Anal. Sci.*, 7, Suppl. (1991) 231.
[2] K.-R. Kim, J.-H. Kim, C.-H. Oh and T.J. Mabry, *J. Chromatogr.*, 605 (1992) 241.
[3] M.G. Horning, W.G. Stillwell, K. Lertratanang and K. Halpaap-Wood, in H. Jaeger (Editor), *Glass Capillary Chromatography in Clinical Medicine and Pharmacology*, Marcel Dekker, New York, 1985, p. 473.
[4] T. Niwa, *J. Chromatogr.*, 379 (1986) 313.
[5] S. Hunt, in G.C. Barrett (Editor), *Chemistry and Biochemistry of the Amino Acids*, Chapman & Hall, New York, 1985, p. 55.
[6] P.M. Smith, *The Chemotaxonomy of Plants*, Edward Arnold, London, 1976, p. 42.
[7] F. Lambein, Y.-H. Kuo, F. Ikegami and I. Murakoshi, in G. Lubec and G.A. Rosenthal (Editors), *Amino Acids*, Escom, Leiden, 1990, p. 27.
[8] G.A. Rosenthal, in G.A. Rosenthal (Editor), *Herbivores, Their Interaction with Secondary Plant Metabolites*, Academic Press, San Diego, CA, 1992, pp. 1–34.
[9] E.A. Bell and P.B. Nunn, *Biologist*, 35 (1988) 39.
[10] S.M. Rothman and J.W. Olney, *Trends Neurosci.*, 10 (1987) 299.
[11] B. Meldrum, *Brain Res. Rev.*, (1993) 293–314.
[12] B. Meldrum and J. Garthwaite, *Trends Pharmacol. Sci.*, 11 (1990) 379.
[13] K.-R. Kim, M.-K. Hahn, A. Zlatkis, E.C. Horning and B.S. Middleditch, *J. Chromatogr.*, 468 (1989) 289.
[14] J.R. Coulter and C.S. Hann, *J. Chromatogr.*, 36 (1968) 42.
[15] M. Makita, S. Yamamoto and M. Kono, *J. Chromatogr.*, 120 (1976) 120.
[16] K.-R. Kim, J.-H. Kim, H.-K. Park and C.-H. Oh, *Bull. Korean Chem. Soc.*, 12 (1991) 87.
[17] H. Do. Rood, *Anal. Chim. Acta*, 236 (1990) 115.
[18] H.M. Liebich, *Anal. Chim. Acta*, 236 (1990) 121.
[19] Y. Okano, T. Kadota, J. Nagata, A. Matsuda, S. Ijima, K. Takahama and T. Miyata, *J. Chromatogr.*, 310 (1984) 251.