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Effect of Selenium Fertilizer on Free Amino Acid Composition of Broccoli (*Brassica oleracea* Cv. Majestic) Determined by Gas Chromatography with Flame Ionization and Mass Selective Detection

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Selenium-enriched broccoli florets, harvested from plants grown on soil fertilized with four levels of sodium selenate, were evaluated for their free amino acid composition using alkylchlorformate derivatization, solid-phase extraction, and GC-FID or GC-MS. The selenium-enriched florets contained 0.4 (control), 5.7 (treatment A), 98.6 (treatment B), and 879.2 (treatment C) μg/g Se (dry weight). Twenty-one free amino acids were identified in the control and all three treatments. The total free amino acid content of the broccoli florets ranged from 178 mmol/kg (dry weight), for the control, to 479 mmol/kg (dry weight), for treatment C. Broccoli from treatment C contained the highest level of Se, had the most total free amino acids, and had an extremely high level of glutamine (Gln) when compared to the control and the other two treatments. In general, the smallest addition of Se to the soil (treatment A) induced increased levels of all detectable amino acids when compared to the control, whereas increased additions of Se (treatments B and C) produced mixed responses. Florets from treatment A contained the highest essential amino acid content.

KEYWORDS: ***Brassica*; broccoli; methiin; free amino acid; selenium; GC-FID; GC-MS**

INTRODUCTION

Recently, there has been renewed interest in eating a healthy diet rich in fruits and vegetables. This trend has emphasized the advantage of eating broccoli (genus *Brassica*, family Cruciferae also known as Brassicaceae), which has been found to contain compounds (carotenoids, chlorophyll, phenolics, vitamins A, C, and E, dietary fiber, selenium-containing amino acids, and glucosinolates) that possess anticarcinogenic properties (*1*, *2*).

Selenium (Se) is an essential micronutrient of human nutrition that has been implicated in reducing the risk of prostate cancer in men (*3*). The current Recommended Dietary Allowance (RDA) for both men and women is 55 μg (0.7 μmol) of Se per day. The Food and Nutrition Board recommends a daily intake level of up to 400 μg for adults. Although rare in the United States, there are reported cases of Se toxicity (*4*, *5*).

Se-enriched broccoli has been demonstrated to reduce the number and size of colon (fed 1−2 μg of Se/g of diet) and mammary (fed 3 μg of Se/g of diet) tumors in rats (*6*, *7*). Se enrichment may provide additional health-beneficial compounds to those naturally present in broccoli. Se absorbed by broccoli is often sequestered in methionine (Se-methionine) and in amino acid secondary metabolites such as Se-methyl-Se-cysteine (*8*). In addition, amino acids are precursors for glucosinolate production (*9*). Robbins et al. (*10*) showed that Se fertilization led to altered concentrations of phenolic acids, glucosinolates, and sulfurophanes in broccoli. No research, however, has examined how Se fertilization influences the free amino acid composition of broccoli.

Numerous chromatographic methods have been published for determining free amino acids in biological materials (*11*−*14*). A method employing alkylchlorformate derivatization, solid-phase extraction (SPE), and gas chromatography−flame ionization detection or −mass spectrometry (GC-FID or GC-MS) has been recently used in the analysis of honey (*15*) and garlic (*16*) and is based upon a method developed by Hušek (*17*). Samples having Se- and sulfur-containing amino acids have been successfully analyzed with this method, in addition to common amino acids (*15*, *17*, *18*). The method is fast and easily implemented using a commercially available kit (Phenomenex, Torrance, CA). A disadvantage of the method is that it cannot be used for the determination of arginine, which binds irrevers-

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ibly to the GC column. This is not a disadvantage for broccoli, however, as the reported levels of arginine have been relatively low (*14*, *19*).

This study examines the free amino acid composition of broccoli grown in a Se-enriched soil. The objectives were to evaluate the use of alkylchloroformate derivatization and GC-FID or GC-MS as methods for the qualitative and quantitative determination of free amino acids and to take an initial look at the effect of different levels of Se fertilization (0.4, 5.7, 98.6, and 879.2 ppm) on the composition of free primary (genetically encoded) and secondary metabolite amino acids in broccoli florets.

MATERIALS AND METHODS

Plant Material. Freeze-dried broccoli (*Brassica oleracea* cv. Majestic) samples, which had been treated with four different levels of Se fertilization (*10*), were used in this study. After heads began forming, 10 mL of 0 mM (control) and 0.17, 0.52, and 5.2 mM sodium selenate solution (treatments A, B, and C, respectively) were applied to the developing plants every other day for 8 days, and then 20 mL was applied every other day for two additional applications. The sodium selenate solution treatments resulted in 0.4 (control), 5.7 (treatment A), 98.6 (treatment B), and 879.2 (treatment C) μg/g of Se (dry weight) in the broccoli florets. All plants were grown in a greenhouse. Broccoli florets were harvested (before flower development), quartered, and frozen at −80 °C. Frozen broccoli florets were then freeze-dried and coarsely ground in a food processor. Ground samples were kept frozen at −20 °C until extraction. Details of how the Se concentration was determined by hydride generation atomic absorption spectroscopy can be found in Finley et al. (*20*).

Reagents and Standards. *S*-Methyl-L-cysteine sulfoxide (methiin) and cysteine were purchased from Research Organics (Cleveland, OH). Se-Methylseleno-L-cysteine, Se-methionine, and l-(+)-*S*-allylcysteine sulfoxide (alliin) were purchased from LKT Laboratories (St. Paul, MN). Solid-phase extraction sorbent tips, amino acid standards, and all reagents used for the SPE and derivatization steps were included with "EZ:faast GC-FID for free amino acid analysis" and "EZ:faast GC-MS for free amino acid analysis" kits, which were purchased from Phenomenex. All gases used in this study were purchased from Airgas (Hyattsville, MD) and were of chromatographic purity. All other chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Extraction. Coarsely ground freeze-dried broccoli samples were pulverized with a coffee grinder and passed through a screen (sieve number 20, particle size < 825 μm). The powder (200 mg) was then extracted with 5 mL of distilled water and sonicated for 30 min with a Branson Ultrasonicator (Branson Cleaning Equipment Co., Shelton, CT) at 40 °C. This broccoli slurry was then centrifuged (low-speed centrifuge for 10 min) and the supernatant collected. The pellet was re-extracted three times with 2.5 mL of distilled water, with the supernatant being collected each time. For the initial extraction efficiency experiments only 0.4 ppm of Se broccoli sample was used, and the four supernatants were collected and analyzed separately. For the actual analysis of the broccoli grown at each level of Se enrichment, all four supernatants were combined. All extracts were then filtered through a 0.45 μm pore size PP filter membrane Whatman filter (Whatman International, Clifton, NJ). Filtered extracts were stored at −70 °C until further analysis. Extraction of each enrichment levels was repeated three times.

SPE and Derivatization. Filtered broccoli extract (200 μL) and 200 μL of internal standard (norvaline at a concentration of 200 μmol/L) were placed in a glass vial and slowly absorbed through a 40 μL resin-packed sorbent tip (*21*) by a 1.5 mL syringe. The resin was then rinsed with the washing solution (200 μL). Residual liquid was removed by pulling air through the sorbent tip. The sorbent particles were collected in the glass vial by the eluting medium (200 μL). Reagent 4 (50 μL) was added to the glass vial and vortexed for 5 s. The reaction was allowed to proceed for 2 min, and the liquid was then re-emulsified by vortexing for 5 s and allowed to proceed for another 1 min. Reagent 5 (100 μL) was added and then vortexed for 5 s, and the reaction was left to proceed for another 1 min. FID reagent 6 (100 μL) was added and vortexed for 5 s, and then the top organic layer was transferred into a GC vial containing an insert for the FID analysis.

For MS analysis, the organic layer was removed without the addition of FID reagent 6 and placed in a GC vial with insert. The organic layer was slowly evaporated under a gentle stream of nitrogen by an N-EVAP analytical evaporator (Organomation Associates, Inc., Berlin, MA). The residue was then reconstituted with MS reagent 6 (100 μL). Cleaned and derivatized samples were immediately analyzed by GC-FID or GC-MS.

***S*-Alk(en)ylcysteine Sulfoxide Determination.** Additional reduction steps were carried out after the derivatization process to determine the presence of *S*-alk(en)ylcysteine sulfoxide in the broccoli samples by GC-FID and GC-MS. The reduction step was performed as described by Kubec et al. (*22*). Briefly, the derivatized sulfoxide-containing amino acids within the broccoli samples were reduced by the addition of a sodium iodide solution (200 μL of 1 g of sodium iodide/mL) and acetyl chloride (50 μL). This mixture was incubated at room temperature for 2 h. Stannous chloride crystals were added to remove excess iodine after the incubation period. The reduced-derivatized sulfoxide amino acids were then extracted with dichloromethane (400 μL). Reduced samples were analyzed using the identical GC-FID and GC-MS condition as the free amino acid determination described in the next section. A garlic sample was also extracted, derivatized, and reduced to demonstrate the separation of alliin to methiin. Details of the sample preparation for the garlic samples can be found in Lee and Harnly (*16*).

Determination of Free Amino Acid Composition. For both the FID and MS analysis, a Zebron ZB-PAAC-MS 10 m × 0.25 mm column (stationary phase information was proprietary) from Phenomenex was used.

GC-FID Conditions. A Hewlett-Packard (HP) 6890 Network GC system (Wilmington, DE) equipped with a HP 7683 series injector and a flame ionization detector was used to analyze the derivatized free amino acids. The helium carrier gas flow was a constant at 1.8 mL/min during the run, and the column head pressure was 9.33 psi. The GC oven temperature was programmed to initially hold at 90 °C for 1 min, then raised to 140 °C (15 °C/min), and finally increased to 320 °C (35 °C/min) and held for 1 min. The inlet temperature was 250 °C. The detector was at 320 °C. A 2 μL sample was injected in split mode (15:1, v/v).

GC-MS Conditions. A Hewlett-Packard (HP) 6890 Network GC system (Wilmington, DE) coupled to a HP 5973 mass spectrometer was used to confirm the identification of the free amino acids present in the standards and samples. The helium carrier gas was a constant at 1.8 mL/min. The oven temperature program was identical to the GC-FID oven program. The injection port temperature was 250 °C. The MS temperatures were set at 250 °C for the ion source, 180 °C for the quadrupole, and 310 °C for the auxiliary. The scan range was set to 30−500 (3.15 scans/s). The injection volume was 2 μL. Injection was done in split mode (15:1, v/v).

Statistical Analysis. The significant difference among control and treatments A, B, and C was determined at the 95% confidence level using the Tukey honest significant difference (HSD) test. Statistica for Windows version 7.0 was used (StatSoft, Inc., Tulsa, OK). Values expressed as millimoles per kilogram of dry weight were used to perform the statistical analysis.

RESULTS AND DISCUSSION

Table 1 lists (in order of elution) the mass fragment ions of the derivatized free amino acid standards and internal standard (norvaline) that were used to confirm the identification of the peaks. Se-Methylseleno-L-cysteine and Se-MET standards were included because these compounds have been reported in Se-enriched broccoli (*23*). **Figure 1** shows the GC-FID chromatogram of the amino acid standards from **Table 1** and the mass spectra of Se-methylseleno-L-cysteine and Se-MET by GC-MS. **Table 2** shows the extraction efficiency using water as the solvent. More than 87% of the total free amino acids were

Table 1. 29 Amino Acid Standards Used in This Study Presented in the Order of Elution with Their Chemical Name, Abbreviation, and Mass Fragment Ions[a]

elution order	chemical name	abbreviation	mass fragment ions
1	*alanine*	*Ala*	*130, 88*
2	sarcosine	Sar	130, 217
3	*glycine*	*Gly*	*116, 207*
4	α-aminobutyric acid	Aba	144, 102
5	*valine*	*Val*	*158, 116*
6	*β-aminobutyric acid*	*BAIB*	*158, 116*
7	internal standard (norvaline)	IS	158, 72
8	*leucine*	*Leu*	*172, 86*
9	allo-iosleucine	AILE	172, 130
10	*isoleucine*	*Ile*	*172, 130*
11	*threonine*	*Thr*	*160, 101*
12	*serine*	*Ser*	*146, 203*
13	*proline*	*Pro*	*156, 243*
14	*asparagine*	*Asn*	*155, 69*
15	Se-methylseleno-L-cysteine	not used	311, 224
16	*aspartic acid*	*Asp*	*216, 130*
17	*methionine*	*Met*	*203, 277*
18	4-hydroxyproline	Hyp	172, 86
19	Se-methionine	Se-Met	325, 265
20	*glutamic acid*	*Glu*	*230, 170*
21	*phenylalanine*	*Phe*	*206, 190*
22	α-aminoadipic acid	AAA	244, 98
23	*glutamine*	*Gln*	*84, 187*
24	ornthine	Orn	156, 70
25	*lysine*	*Lys*	*170, 128*
26	*histidine*	*His*	*282, 168*
27	*tyrosine*	*Tyr*	*206, 107*
28	*trptophan*	*Trp*	*130*
29	cystine	C–C	248, 216

[a] Compounds in italic type were found in the Se-enriched broccoli samples evaluated in this study.

removed with two extractions. In this study, the supernatants from all four extractions were combined for the free amino acid analysis.

Figure 2 shows the free amino acid chromatograms of the broccoli florets grown with different levels of Se. The internal standard (peak 7, norvaline) demonstrates that scalings for all four chromatograms are very close to the same. All of the unlabeled peaks were carefully examined by MS and found to be byproducts of the sample preparation process (cleanup and derivatization). Twenty-one free amino acids (not including peak 7, the internal standard) can be seen in the Se-enriched broccoli samples (*S*-methylcysteine sulfoxide, Ala, Gly, Val, BAIB, Leu, Ile, Thr, Ser, Pro, Asn, *S*-methylcysteine, Asp, Met, Glu, Phe, Gln, Lys, His, Tyr, And Trp) in **Figure 2**. Two amino acid peaks were observed for which there was no appropriate internal standard or purified standard commercially available and therefore were not quantified (**Table 1**): *S*-methylcysteine sulfoxide (methiin, peak *) and *S*-methylcysteine (peak 30). *S*-Methylcysteine was detected and identified (by GC-MS) in all four broccoli samples but was not quantified because there was no commercially available standard.

S-Methylcysteine was previously identified in broccoli by Murcia et al. (*19*). Cai et al. (*23*) reported *S*-methylcysteine to be the major sulfur-containing amino acid in their Se-enriched broccoli sample, which was determined by ethylchloroformate derivatization with GC−atomic emission detection (GC-AED). Cysteine was not present in the suite of standards listed in **Table 1** because it was not available in a purified form. On the basis of an impure standard, it was determined that cysteine was not present in the samples analyzed in this study. Cysteine was not detected in broccoli samples examined by other researchers (*14*, *19*).

Derivatized *S*-methylcysteine sulfoxide (methiin) was found to decompose under the GC conditions used for these analyses and would randomly appear as one or two peaks (data not shown). Kubec et al. (*22*) described a reduction step that reproducibly converted *S*-alkylcysteine sulfoxides to *S*-alkylcysteine. This approach was successfully employed for the determination of methiin and alliin in garlic (*22*), in which the *S*-methylcysteine concentration was negligible. **Figure 3** shows the GC-FID profiles for a reduced methiin standard and reduced broccoli (treatment C). The reduced methiin appears, as expected, at the time of the *S*-methylcysteine peak.

Table 2. Percent of Free Amino Acids Removed from 200 mg of Sample with Four Successive Extractions with Water, Assuming All Free Amino Acids Were Extracted by the Fourth Extraction ($n = 3$)[a]

		1st extraction (200 mg/5 mL of water)		2nd extraction with 2.5 mL of water		3rd extraction with 2.5 mL of water		4th extraction with 2.5 mL of water	
peak		av	SD	av	SD	av	SD	av	SD
1	Ala	70.5	1.4	17.4	0.6	8.1	0.5	4.0	0.3
3	Gly	82.7	0.8	15.3	1.9	2.0	1.4	0.0	0.0
5	Val	70.5	1.2	17.3	0.3	8.2	0.5	4.0	0.6
6	BAIB	80.4	11.2	11.8	10.3	6.8	0.7	1.0	1.0
8	Leu	69.8	0.7	17.3	0.2	8.5	0.3	4.4	0.2
10	Ile	70.5	1.2	17.6	0.6	8.1	0.6	3.8	0.4
11	Thr	72.3	0.8	17.3	0.4	7.2	0.4	3.2	0.2
12	Ser	60.8	1.5	18.9	0.9	13.4	0.3	6.9	0.3
13	Pro	71.7	1.1	16.7	0.7	7.8	0.2	3.8	0.2
14	Asn	71.1	0.6	17.6	0.5	7.5	0.2	3.8	0.1
16	Asp	60.7	1.8	27.1	1.1	12.2	1.4	0.0	0.0
17	Met	71.7	0.8	17.2	0.5	7.5	0.2	3.6	0.2
20	Glu	70.7	3.2	17.4	1.0	7.9	1.5	4.0	0.7
21	Phe	74.5	4.9	18.9	0.8	6.6	5.7	0.0	0.0
23	Gln	70.9	2.4	18.7	0.2	7.8	0.5	2.6	1.8
25	Lys	61.2	3.0	17.7	1.5	13.5	4.5	7.7	1.2
26	His	65.1	4.3	19.9	3.4	11.3	1.3	3.8	3.8
27	Tyr	67.8	4.0	17.6	1.0	9.0	1.0	5.6	5.4
28	Trp	61.6	4.4	20.5	2.0	14.3	0.8	3.7	6.4
sum		69.9	1.1	17.7	0.4	8.5	0.5	3.9	0.5

[a] Amino acids are listed in order of elution. SD, standard deviation; av, average.

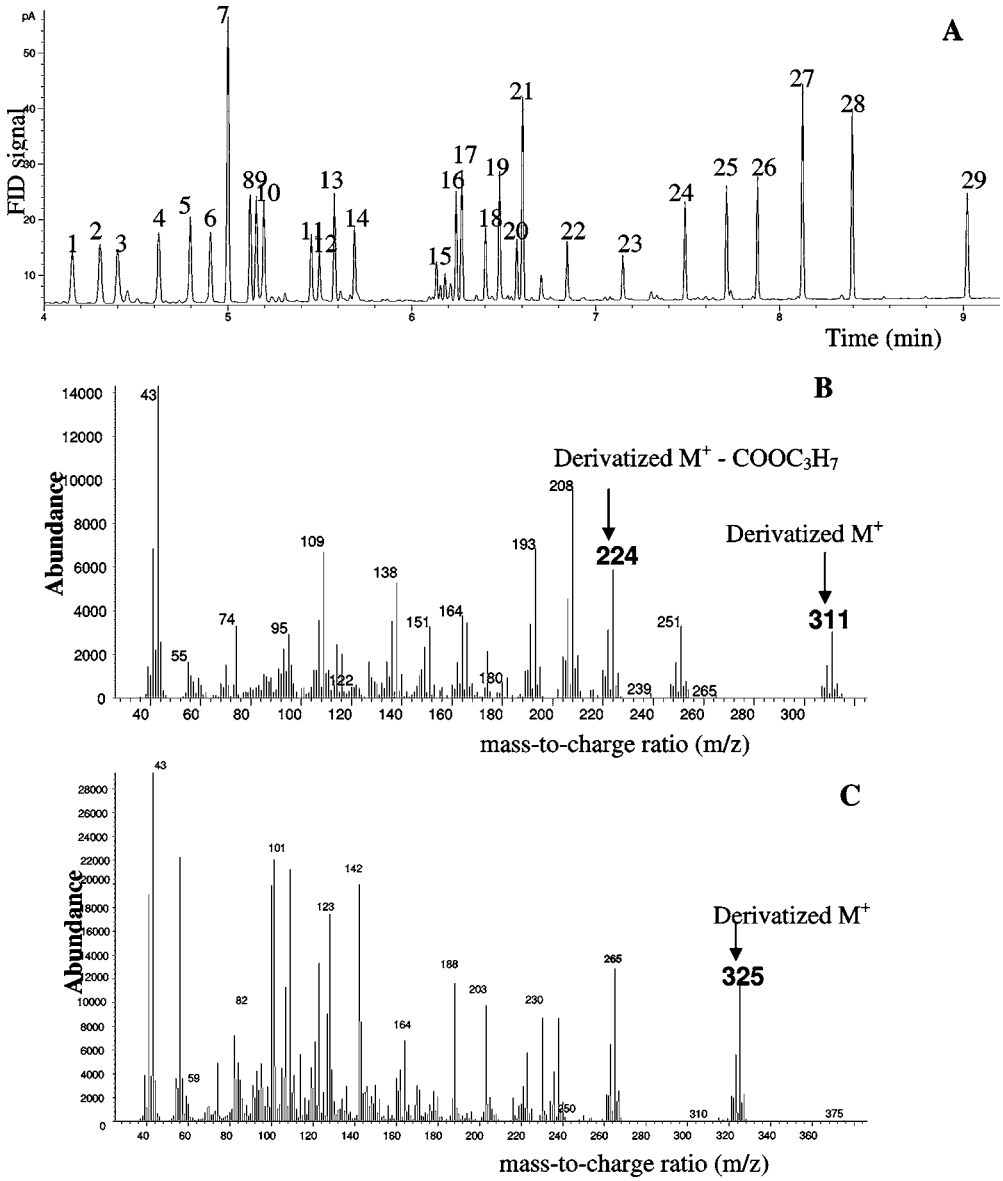

Figure 1. Amino acid profile of the 29 standards obtained by GC-FID (**A**) and the mass spectra of peaks 15 (derivatized Se-methylseleno-L-cysteine, **B**) and 19 (derivatized Se-methionine, **C**) obtained by GC-MS. Methiin was not included in this run, but the decomposed derivatized methiin eluted right before Ala (as observed in **Figure 2**). Abbreviations used are listed in **Table 1**. Peaks: 1, Ala; 2, Sar; 3, Gly; 4, Aba; 5, Val; 6, BAIB; 7, IS; 8, Leu; 9, alle; 10, Ile; 11, Thr; 12, Ser; 13, Pro; 14, Asn; 15, Se-methylseleno-L-cysteine; 16, Asp; 17, Met; 18, Hyp; 19, Se-Met; 20, Glu; 21, Phe; 22, AAA; 23, Gln; 24, Orn; 25, Lys; 26, His; 27, Tyr; 28, Trp; 29, C–C. M^+ = molecular ion.

Broccoli contains measurable quantities of *S*-methylcysteine (**Figure 2**). Consequently, the reduction of the methiin will increase the signal for *S*-methylcysteine. In theory, it should be possible to quantify both by measuring the peak intensity of *S*-methylcysteine with and without reduction. However, there was no commercially available standard for *S*-methylcysteine at the time of this study. Kubec et al. (*24*) reported 2.08 mg/kg methiin (fresh weight) in broccoli using their reduction method, but do not distinguish between methiin and *S*-methylcysteine sulfoxide. Kubec et al. (*24*) used methiin as their primary standard and butylcysteine sulfoxide, which they synthesized, as an internal standard to account for the efficiency of the reduction step.

Table 3 summarizes the measured concentrations for 19 individual free amino acids in the control and treatments A−C. Concentrations are reported as milligrams per gram and millimoles per kilogram, both on a dry weight basis, and standard deviations are reported in units of millimoles per kilogram. The four broccoli samples had total free amino acid concentrations ranging from 178 mmol/kg (dry weight) for the control to 479 mmol/kg (dry weight) for treatment C. The total free amino acid content of the florets from the control (178 mmol/kg) was statistically different from the contents of treatments A and B (385 and 345 mmol/kg, respectively), which were statistically different from the content of treatment C (479 mmol/kg). Treatment C had higher total free amino acid than any of the 11 cultivars of broccoli examined by Gomes et al. (*14*). They reported total free amino acid contents ranging from 177 to 324 mmol/kg (dry weight) for the SK3 and Shogan cultivars, respectively. They determined amino acid concentrations by HPLC after *o*-phthalaldehyde derivatization.

The concentration of individual amino acids as a function of added Se varied considerably (**Table 3** and **Figure 4**). In every case, treatment A produced an increase in the concentration of every detectable amino acid as compared to the control. The responses to treatment B were approximately similar to those for treatment A. Responses to treatment C were the most unpredictable. Most noticeable were the doubling or tripling in

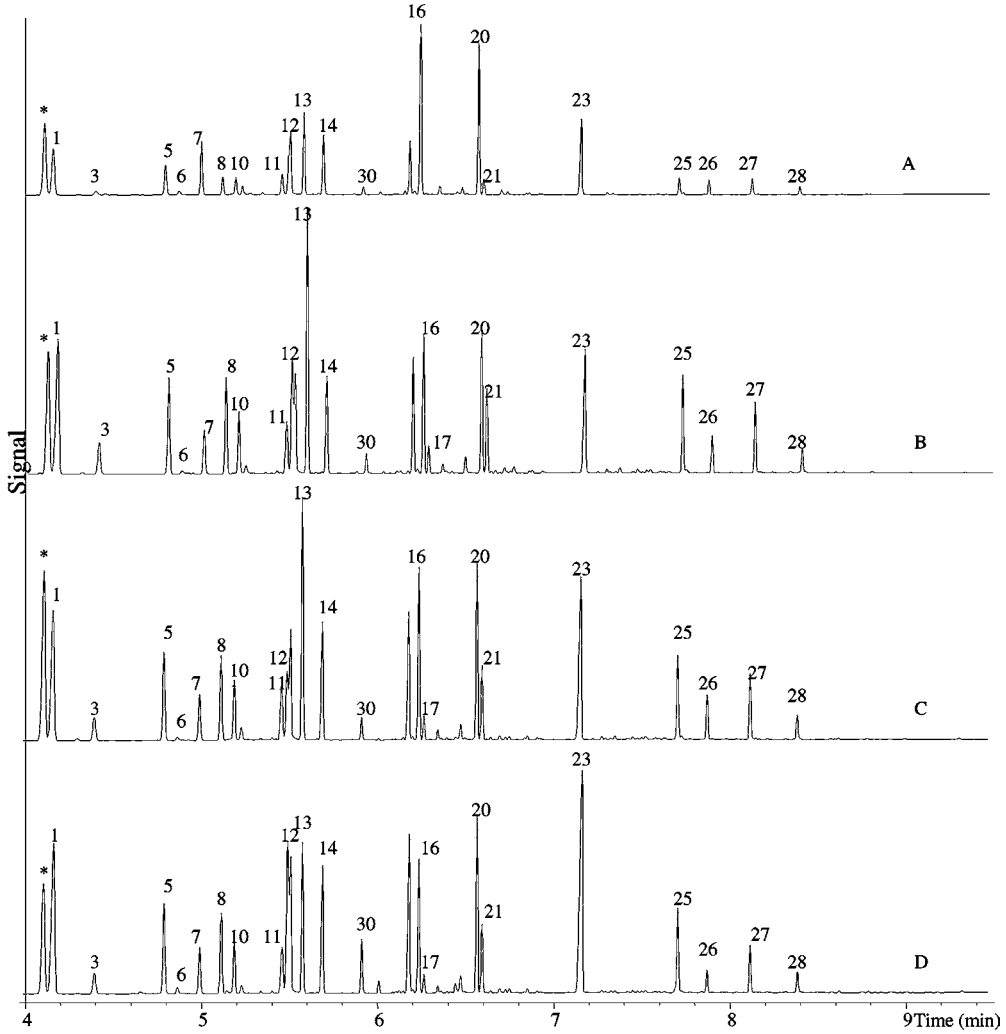

Figure 2. Free amino acid profiles obtained by GC-FID of freeze-dried broccoli florets grown in four different levels of selenium fertilization (**A**, 0.4 ppm, control; **B**, 5.7 ppm, treatment A; **C**, 98.6 ppm, treatment B; **D**, 879.2 ppm, treatment C). Peaks: *, methiin degradation product; 1, Ala; 3, Gly; 5, Val; 6, BAIB; 7, IS; 8, Leu; 10, Ile; 11, Thr; 12, Ser; 13, Pro; 14, Asn; 30, *S*-methylcysteine (confirmed by GC-MS only); 16, Asp; 17, Met; 20, Glu; 21, Phe; 23, Gln; 25, Lys; 26, His; 27, Tyr; 28, Trp.

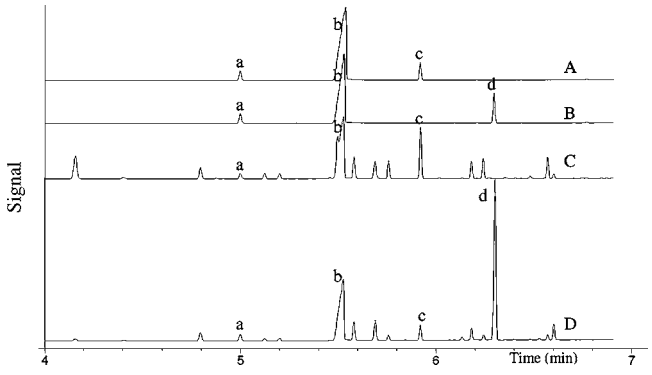

Figure 3. GC-FID profile of derivatized and reduced methiin standard (**A**), alliin standard (**B**), Se-enriched (879.2 ppm) broccoli sample (**C**), and blanched garlic (**D**): (a) IS (norvaline); (b) reduction byproduct; (c) methiin reduced; (d) alliin reduced.

concentration of Gln, Ser, and Ala in treatment C samples when compared to the control.

The varied responses of the individual amino acids produced variation in the major constituents of the florets for the different treatments. The major amino acids present in the control were Glu (23%), Ser (16%), Gln (15%), and Asn (14%); present in treatment A were Ser (13%), Gln (13%), Ala (12%), and Glu (11%); present in treatment B were Gln (17%), Glu (15%), Ala (12%), and Pro (10%); and present in treatment C were Gln (28%), Ser (18%), Glu (11%), and Ala (11%). The concentrations of the six amino acids listed above are traced individually in **Figure 4**. This plot emphasizes the variability of response of the amino acids to the Se treatments. The statistical significance of the different individual amino acid concentrations is provided in **Table 3**. The control and all three treatments had statistically significant different levels of Ala, Gly, Pro, and Met, but they had no significant difference in their His levels. The control had significantly lower levels for Phe and Gln free amino acids when compared to treatment C. The addition of selenium increased the concentrations of Ala, Gly, Val, Leu, Ile, Thr, Met, Phe, Lys, and Tyr in the three treatment samples when compared to the control.

Two amino acids were not detected in this study that have been previously reported. Murcia et al. (*19*) reported 0.06 mg/g of ornithine (fresh weight) and 0.03 mg/g of γ-aminobutyric acid (fresh weight) in their broccoli samples (determined by ion exchange chromatography). Assuming 80% moisture content, the dry weight concentrations would be 0.24 and 0.12 mg/g, respectively. These levels are equivalent to 10 and 5%, respectively, of the total free amino acid concentration. Arginine in these broccoli samples could not be detected due to the

Table 3. Free Amino Acid Composition of the Se-Enriched Broccoli Samples Obtained by GC-FID[a]

	treatment:	control		A		B		C	
	actual Se concn:	0.4 ppm		5.7 ppm		98.6 ppm		879.2 ppm	
	units:	mmol/kg	mg/g	mmol/kg	mg/g	mmol/kg	mg/g	mmol/kg	mg/g
1	Ala	12.40 (± 0.43) a	1.10	45.66 (± 0.43) b	4.07	41.28 (± 0.95) c	3.68	52.25 (± 0.38) d	4.65
3	Gly	0.69 (± 0.07) a	0.05	9.33 (± 0.19) b	0.70	6.22 (± 0.14) c	0.47	5.43 (± 0.03) d	0.41
5	Val	**4.74** (± 0.02) a	0.56	**18.31** (± 0.22) b	2.15	**15.67** (± 0.41) c	1.84	**15.84** (± 0.16) c	1.86
6	BAIB	0.68 (± 0.05) a	0.07	0.60 (± 0.08) ab	0.06	0.49 (± 0.07) b	0.05	1.22 (± 0.06) c	0.13
8	Leu	**2.10** (± 0.04) a	0.28	**14.49** (± 0.13) b	1.90	**11.32** (± 0.05) c	1.49	**11.05** (± 0.17) c	1.45
10	Ile	**2.31** (± 0.01) a	0.30	**10.24** (± 1.33) b	1.34	**8.37** (± 0.51) bc	1.10	**7.54** (± 0.14) c	0.99
11	Thr	**4.50** (± 0.02) a	0.54	**15.20** (± 0.38) b	1.81	**14.23** (± 1.18) bc	1.70	**13.04** (± 0.3) c	1.55
12	Ser	28.48 (± 6.08) a	2.99	51.63 (± 2.31) abc	5.43	28.64 (± 0.79) bc	3.01	87.24 (± 27.48) c	9.17
13	Pro	10.36 (± 0.07) a	1.19	42.75 (± 0.90) b	4.92	34.02 (± 0.45) c	3.92	21.15 (± 0.49) d	2.43
14	Asn	11.02 (± 0.14) a	1.46	21.71 (± 0.19) b	2.87	23.96 (± 0.69) c	3.17	25.40 (± 1.11) c	3.36
16	Asp	24.44 (± 0.63) a	3.65	22.57 (± 0.63) b	3.37	26.47 (± 0.33) c	3.95	20.28 (± 0.79) d	3.03
17	Met	**0.08** (± 0.01) a	0.01	**3.50** (± 0.07) b	0.52	**2.83** (± 0.22) c	0.42	**2.08** (± 0.02) d	0.31
20	Glu	41.15 (± 1.79) a	5.48	44.09 (± 1.43) ab	5.87	51.12 (± 5.11) bc	6.80	54.09 (± 1.28) c	7.20
21	Phe	**1.01** (± 0.01) a	0.17	**7.03** (± 0.14) b	1.16	**5.42** (± 0.10) c	0.90	**5.36** (± 0.07) c	0.89
23	Gln	27.31 (± 1.54) a	3.99	50.08 (± 14.52) a	7.32	57.24 (± 24.46) a	8.37	133.86 (± 2.60) b	19.56
25	Lys	**2.32** (± 0.07) a	0.34	**13.02** (± 0.61) bc	1.90	**8.00** (± 5.26) abc	1.17	**11.17** (± 0.17) bc	1.63
26	His	2.44 (± 0.05) a	0.38	5.97 (± 0.39) a	0.93	4.70 (± 4.07) a	0.73	4.40 (± 0.57) a	0.68
27	Tyr	**1.44** (± 0.15) a	0.26	**5.69** (± .64) b	1.03	**3.77** (±1.77) ab	0.68	**4.94** (± 1.44) b	0.89
28	Trp	1.05 (± 0.01) a	0.21	2.60 (± 0.09) b	0.53	0.93 (± 0.31) a	0.19	2.37 (± 0.06) b	0.48
	total	178.53 (± 8.13) a	23.03	384.46 (± 13.68) b	47.81	344.69 (± 44.13) b	43.61	478.70 (± 31.30) c	60.67
	r-methiin	9.69		18.50		18.72		15.22	

[a] Individual amino acid concentrations expressed as mmol/kg and mg/g (both dry weight). Values in bold type indicate essential amino acids. Amino acids are listed in the order of elution. r-Methiin is methiin that has been derivatized and reduced. Because no internal standards were available (for the reduction step), the values listed are peak areas. Individual and total amino acid values in the same row, with different letters (expressed as mmol/kg of dry weight) are significantly different ($p < 0.05$). Values in parentheses are standard deviations ($n = 3$).

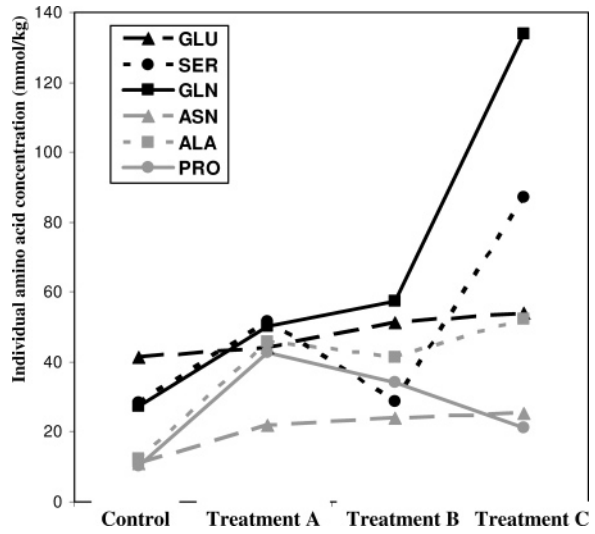

Figure 4. Plot of six individual amino acid (Glu, Ser, Gln, Asn, Ala, and Pro) concentrations as a function of Se treatments.

permanent absorption of the arginine imino group onto the column, a noted disadvantage of this method (*17*). Gomes et al. (*14*) reported that their broccoli samples contained 2.56−11.50 mmol/kg (dry weight) of arginine. These levels represent 2.5−10% of the total free amino acid concentration. Combined, these three amino acids could increase the total free amino acid concentration by as much as 20%.

The broccoli samples contained eight essential amino acids: Val, Leu, Ile, Thr, Met, Phe, Lys, and Tyr (in order of elution). Growing broccoli with treatment A, B, or C increased its essential amino acid concentration (**Table 3**, bold type). Treatment A contained the most essential amino acids, followed in order by treatment C, treatment B, and the control. The major essential amino acid present in all of the samples was Val. Munshi et al. (*25*) have reported an increase of essential amino acids in potatoes grown in Se-supplemented soil.

Broccoli may tolerate high Se by forming Se-containing amino acids as a means of detoxification (*8*). The Se-containing amino acids identified in broccoli grown in the Se-enriched environment of treatment B were Se-methylseleno-cysteine (63%), selenocysteine (11%), and selenomethionine (5%) (*23*). These results were obtained by derivatizing the Se compounds and separation and detection with GC-AED using a microwave-induced plasma device (*23*). The percentages are based on peak areas because the determinations were strictly qualitative (*23*). GC-AED, which detects Se emission, is more sensitive and selective than GC-FID or GC-MS for the determination of Se-containing amino acids (*11*). A more popular method for the detection of Se-containing compounds is liquid chromatography−inductively coupled plasma mass spectrometry (LC-ICP-MS) (*26*−*28*). For both instruments, the detection device destroys the organic compound and the only basis for compound identification is the retention time, which must be matched with known standards.

No Se-containing amino acids were detected in this study. One possibility is that the method is not sufficiently sensitive. Nozal et al. (*15*) reported amino acid detection limits (for the same method used in this study) of 0.11−1.8 mg/L by GC-FID and 0.001−0.29 mg/L by GC-MS. Haberhauer-Troyer et al. (*11*) reported a limit of detection for the Se-containing amino acids of 0.4 mg/L using their derivatization, extraction, GC-AED method. They proposed that the derivatized Se-containing amino acids decomposition can be controlled by inlet temperature, oven temperature, and liner used. Roberge et al. (*26*) reports in detail how the pH of the extraction solution and filtration influence these Se compounds found in Se-enriched broccoli. The peaks for Se-methylseleno-cysteine and selenomethionine that were obtained in this study (**Figure 1**) correspond to detection limits of roughly 43 and 140 mg/L, respectively, well above the levels

reported in the other studies. It seems likely that significant degradation took place.

In conclusion, Se-enriched broccoli contains more Se and higher levels of essential amino acids, total free amino acids, and methiin than broccoli grown under normal conditions and could potentially provide additional health-beneficial compounds. The SPE, alkylchloroformate derivatization, and GC-FID or GC-MS analysis takes <20 min, which makes this procedure quick and simple for analyzing common free amino acids present in broccoli. The disadvantage of this method is the inability to analyze arginine. Detection of secondary metabolite amino acids was complicated by the lack of standards and degradation of the S- and Se-containing compounds.

ABBREVIATIONS USED

SPE, solid-phase extraction; GC-FID, gas chromatography with flame ionization detection; GC-MS, gas chromatography with mass spectrometry.

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