SUPPLEMENTARY MATERIAL

Profiling of Organosulfur Compounds using HPLC-PDA and GC/MS system and antioxidant activity in hooker chive (*Allium hookeri*)

Sunyoung Kim, Dan-Bi Kim, Sanghee Lee, Jisu Park, Dongbin Shin, Miyoung Yoo*
Food Analysis Center, Korea Food Research Institute, 516 Backhyun-dong, Bundang-gu, Sunnam-si, Gyeonggi-Do, 463-746, Republic of Korea

* Corresponding author: Dr. Miyoung Yoo. E-mail: myyoo@kfri.re.kr

Division of Food Analysis Center, Korea Food Research Institute, 65, Anyangpangyo-ro 1201beon-gil, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

Abstract

This study investigates the comprehensive organosulfur compounds and evaluation of antioxidant activities in *Allium hookeri*, called hooker chive. The non-volatile and volatile organosulfur compounds were determined by high performance liquid chromatography-photodiode array detector (HPLC-PDA) and gas chromatography-mass spectrometry (GC-MS), respectively. Among 11 non-volatile organosulfur compounds, methiin and cycloalliin were major compounds. A total of 42 volatile compounds were identified and allyl methyl sulfides and dimethyl sulfides were primarily volatiles. The total phenols ranged from 19.32 to 71.19 mg gallic acid equivalents (GAE)/g. The antioxidant capacities expressed IC₅₀ values ranged from 9.59 to 38.54 mg/ml for DPPH radical scavenging activity and from 0.39 to 2.36 for ferric reducing antioxidant power assay (FRAP). The results were proposed as useful tools for evaluating the nutraceutical of hooker chive.

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Keyword: *Allium hookeri*, organosulfur compound, total phenolic, antioxidant

Experimental

Chemicals

Alliin and methiin were purchased from LKT Laboratories (Saint Paul, MN, USA) and cycloalliin, GSMC, GSPC and SPC were obtained from Medigen (Daejeon, Korea). GSAC and allicin were purchased from the United States Pharmacopeia (Rockville, MD, USA). Heptane sulfonic acid and sodium dihydrogen phosphate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Wako Hayashi Pure Chemical Industries, Ltd. (Tokyo, Japan), respectively. Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile was obtained from JT Baker (Phillipsburg, NJ, USA) and the water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA, USA). All other chemicals and solvents were HPLC grade.

Sample preparation for non-volatile organosulfur compounds

The domestic samples, roots and leaves, were harvested in 2013 and were directly purchased from cultivator of Cheongju province, Korea. Imported samples from Myanmar were obtained by Oracom Company (Seoul, Korea). The roots were blanched for 7 min at 80°C for an enzyme inactivation of hooker chive. Then, the samples were crushed using DH 850 laboratory blender (Oscar, Seoul, Korea) for determination of organosulfur compounds. For the measurement of allicin, fresh roots were also prepared by blending (Oscar, Seoul, Korea). The leaves were obtained in dried condition. Then, dried leaves were blended to a fine powder. All samples were packed by vacuum film and stored at -80 °C before extraction and analysis. The extraction of hooker chive was based on the method of Yoo et al. (2010) Five g of a samples was added to 70 mL of water in a 100 mL volumetric flask. The mixture was vortexed for 1 min using a vortex mixer (Thermo Scientific, Waltham, MA, USA) and sonicated for 10 min at room temperature. Additional water was added to the mixture to bring the volume to 100 mL. The extract was filtered by a 0.2 µm membrane syringe filter (Sartorius Stedium Biotech, Geottingen, Germany) and analyzed by HPLC-PDA system.

Analytical conditions for non-volatile organosulfur compounds

The organosulfur compounds were measured using the published HPLC methods.^{22,23} The analytical conditions for organosulfur compounds except methiin and cycloalliin were measured using the method of Yoo et al. (2010) A Waters HPLC- Photodiode array detector (PDA) system (Waters, Milford, MA, USA) was used for organosulfur compounds analysis. The chromatographic separation was carried out using a Hypurity Elite C18 column (150 mm × 3 mm, 3 µm, Thermo Scientific, Waltham, MA, USA) with a C18 guard column cartridge (10 µm, µBondapak^{TM}, Wexford, Ireland) at 38°C, detection was at 208 nm. The flow rate was 0.4 mL/min, and the injection volume was 10 µL. The mobile phase consisted of 20 mM sodium dihydrogen phosphate + 10 mM heptane sulfonic acid,

pH 2.1 (adjusted with 80% orthophosphoric acid, mobile phase A), and Mobile phase A in acetonitrile (50:50, v/v). The following gradient system was used: 100% A (0 min), 70% A (5 min), 46% A (25 min), 0% A (26 min), 0% A (28 min), 100% A (30 min) and 100% A (50 min).

The analysis condition of methiin and cycloalliin was performed according to a method described by Lee et al. (2013) The HPLC system was a Shiseido SP[^]LC chromatographic system equipped with an SP3201 pump, an SP3023 autosampler system and a programmable SP3202 photodiode array detector (PDA) system (Shiseido, Tokyo, Japan). The detection set at 210 nm and the column used was a Shodex Asahipak NH2P-50 4E column (250 mm × 4.6 mm, 3 μm, Showa Donko Co., Tokyo, Japan). The mobile phase consisted of 0.2% phosphoric acid in distilled water as mobile phase A) and 0.2% phosphoric acid in acetonitrile as mobile phase B, respectively. The gradient system used was 16% A (5 min), 20% A (15 min), 16% A (20 min) and 16% A (30 min). The flow rate was kept at 1.5 mL/min, and the sample injection volume was 10 μL.

Sample preparation and analytical conditions for volatile organosulfur compounds

The fresh roots of hooker chive were freeze-dried. The leaves were obtained in dried conditions. The dried samples were weighted, blend to a fine powder and stored at -80°C before extraction and analysis. For extraction, solid-phase microextraction (SPME) was used to isolate the volatile organosulfur compounds of hooker chive. One g of sample was mixed with 4 mL of distilled water in 20 mL vials with a silicone layer/PTFE lamination (Gerstel, Müllheimander Ruhr, Germany) and maintained at 40°C for 30 min to reach an equilibrium state. The volatile compounds were absorbed on to an SPME fiber coated with 50/35 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) for 30 min.

For an analysis of volatile compounds, GC/MS was performed using a HP 6890 series gas chromatograph HP 5973 with a quadruple mass selective detector (Agilent Technologies, Palo Alto, CA, USA). The column used was a DB-WAX column (60 m in length, 0.32 mm i.d., 0.25 μm film thickness, Agilent Technologies Inc., Palo Alto, CA, USA). The analytes were thermally desorbed at 220°C for 5 min, and desorption of the fiber was at a split ratio of 1/10. The GC oven temperature was held at 40°C for 5 min and then raised to 200°C at a rate of 3°C/min. Helium, as a carrier gas, was run at a constant 1.0 mL/min. Mass spectra were obtained at 70 eV through electron ionization (EI) and the temperature of ion source was set at 230°C. The mass scanning range was 35 to 350 (m/z). The identification of volatile compounds was confirmed by comparing the mass spectral data with the Wiley 275&7N mass spectral database (Hewlett Packard, Vienna, Austria).

Determination of total phenolics and antioxidant activities

The total phenolics content was evaluated by a colorimetric analysis using Folin-Ciocalteu's reagent (Singleton & Rossi 1965). Each sample extract (0.2 mL) was mixed with 2.6 mL of distilled water.

Then, 0.2 mL of Folin-Ciocalteu's reagent was added to the mixture and vortexed. Two milliliters of 7% Na₂CO₃ solution was added after 6 min. After 90 min at 23°C, the absorbance was measured at 750 nm. Distilled water was used as a blank. The total phenolics content in hooker chive was calculated by mg gallic acid equivalents (GAE)/g dry weight.

The antioxidant activities of hooker chive were determined using DPPH radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP). The DPPH radical scavenging activity was determined according to a method using Clarke et al. (2013) with some modifications. A 100 µM solution of DPPH radical was prepared in 80% (v/v) aqueous methanol. Fifty microliters of sample extract was added to 2.95 mL of the methanolic DPPH solution. The radical solution was added a sample extract for 30 min. Subsequently, the decreased absorbance of the solution was measured at 517 nm. Aqueous methanol (80%) was used as a reference. The antioxidant capacity of hooker chive was expressed as IC₅₀, the amount of sample extracted into the solution was necessary to decrease the initial DPPH concentration by 50%, which was derived from the percent scavenging ability vs. the concentrations.

FRAP activity was modified according to the method of Ou et al. (2002). The working FRAP reagent was prepared before use by mixing of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM FeCl₃·6H₂O solution in proportions of 10:1:1 (v/v/v), respectively. Then, sample (0.5 mL) was added with distilled water (0.15 mL) and the working FRAP reagent (1.5 mL). The mixture was incubated at 37 °C for 4 min, and the absorbance was measured at 593 nm.

Statistical analysis

All experiments were performed in triplicate. Statistical tests were performed using SAS software version 8.2 (SAS Institute, Inc., Cary, NC, USA). Significant differences were verified by Duncan's Multiple Range Test at a 95% confidence level.

Table S1. The contents of non-volatile organosulfur compounds in hooker chive.

Non-volatile organosulfur compounds	Abbreviations	Concentration (mg/g of dry weight)		
		Roots		Leaves
		Cheongju	Myanmar	
γ -L-glutamyl-S-allyl-L-cysteine	GSAC	0.34±0.01b ¹⁾	0.38±0.02b	2.63±0.15a
γ -L-glutamyl-S-(trans-1-propenyl)-L-cysteine	GSPC	0.78±0.02b	1.67±0.08a	0.24±0.01c
γ -L-glutamyl-S-methyl-L-cysteine	GSMC	0.13±0.00b	0.17±0.00b	2.05±0.20a
γ -glutamyl-phenylalanine	γ GPA	tr ²⁾	tr	0.15±0.01a
(+)-S-(trans-1-propenyl)-L-cysteine	TPC	0.03±0.00b	0.03±0.01b	0.57±0.03a
(+)-S-methyl-L-cysteine	SMC	tr	0.02±0.00b	0.71±0.04a
(+)-S-allyl-L-cysteine sulfoxide	Alliin	3.66±0.05b	4.02±0.05a	n.d. ²⁾
(+)-S-(trans-1-propenyl)-L-cysteine sulfoxide	Isoalliin	tr	tr	0.21±0.01a
(+)-S-methyl-L-cysteine sulfoxide	Methiin	3.99±0.17c	6.39±0.21a	5.17±0.51b
(3 <i>R</i> ,5 <i>S</i>)-5-methyl-1,4-thiazane-3-carboxylic acid	Cycloalliin	1.31±0.08b	1.48±0.07b	11.62±1.26a
S-allyl 2-propene-1-sulfinothioate	Allicin	0.35±0.02a	0.30±0.00b	n.d.

¹⁾ Values are shown as means ± SD of triplicates; significant differences (p<0.05) between the roots and the leaves using Duncan's Multiple Comparison Test between the samples having a different letter in a row.

²⁾ tr, trace (<0.01 mg/g dry wt.); n.d., not detected.

Table S2. Identification of volatile compounds in hooker chive.

Peak No.	RI	Compound	MF ³⁾	MW ³⁾	Peak area (means ± SD) ¹⁾		
					Roots		Leaves
					Cheongju	Myanmar	
Aldehydes							
A1	<800	acetaldehyde	C2H4O	44	522±88b ²⁾	865±213a	319±36b
A2	910	2-methyl butanal	C5H10O	86	n.d ^{d3)}	n.d	183±27a
A3	914	3-methyl butanal	C5H10O	86	n.d	n.d	394±11a
A4	1036	2-butenal	C4H6O	70	227±16b	1476±199a	343±2b
A5	1078	hexanal	C6H12O	100	575±39b	856±41a	525±24b
A6	1090	2-methyl-2-butenal	C5H8O	84	173±20b	75±6c	855±41a
A7	1155	2-methyl-2-pentenal	C6H10O	98	597±21b	205±7c	1171±4a
A8	1393	nonanal	C9H18O	142	n.d	n.d	225±6a
A9	1400	2,4-hexadienal	C6H8O	96	n.d	240±30a	175±12b
Ketones							
K1	1337	6-methyl-5-hepten-2-one	C8H14O	126	n.d	n.d	332±7a
K2	2000	5-methyl-2-hexyl-(2H)-3-furanone	C11H18O2	182	947±96b	4699±164a	n.d
K3	2219	5-methyl-2-octyl-(2H)-3-furanone	C13H22O2	210	1240±89a	851±146b	n.d
Sulfides							
S1	<800	carbon disulfide	CS2	75	86±9b	261±43a	232±45a
S2	926	methyl thiirane	C3H6S	79	396±47a	377±75a,b	280±22b
S3	951	methyl allyl sulfide	C4H8S	88	110±4b	240±14a	n.d
S4	1067	dimethyl disulfide	C2H6S2	93	1161±34c	4825±267a	2051±120b
S5	1103	allyl propyl sulfide	C6H12S	116	87±26b	166±2a	n.d
S6	1144	allyl sulfide	C6H10S	114	121±8b	250±17a	n.d
S7	1229	methyl propyl disulfide	C4H10S2	122	1345±41b	4699±110a	962±40c
S8	1262	(Z)-methyl propenyl disulfide	C4H8S2	120	1528±83c	5108±160a	1923±46b
S9	1279	methyl allyl disulfide	C4H8S2	120	10383±328b	26776±602a	795±26c
S10	1287	(E)-methyl propenyl disulfide	C4H8S2	120	7371±480b	9510±206a	7693±215c
S11	1316	methyl 1-propenyl disulfide	C4H8S2	120	78±4c	124±17b	160±6a
S12	1379	dimethyl trisulfide	C2H6S3	125	17559±520b	43051±451a	11100±364c
S13	1413	(Z)-propenyl propyl disulfide	C6H12S2	148	451±46c	821±74a	629±64b
S14	1430	allyl propyl disulfide	C6H12S2	148	8103±311b	13515±526a	260±34c
S15	1437	(E)-propenyl propyl disulfide	C6H12S2	148	1975±165b	5010±241a	1001±87c
S16	1466	C6H10S2	C6H10S2	146	3954±328b	5246±207a	453±44c
S17	1482	allyl disulfide	C6H10S2	146	22836±909b	35900±1011a	n.d
S18	1592	methyl allyl trisulfide	C4H8S3	151	57936±4108b	83795±4244a	1766±145c
S19	1661	methyl methylthiomethyl disulfide	C3H8S3	139	1513±67b	4727±194a	107±6c
S20	1672	diisopropyl trisulfide	C6H14S3	182	928±126a	797±134a	177±4b
S21	1795	allyl trisulfide	C6H10S3	177	41711±4264a	30159±811b	n.d

(continued)

Peak No.	RI	Compound	MF	MW ⁴⁾	Peak area (means ± SD)		
					Roots		Leaves
					Cheongju	Myanmar	
Vinyldithiins							
V1	1739	3-vinyl-4H-1,2-dithin	C6H8S2	144	5672±208b	8547±143a	n.d.
V2	1847	2-vinyl-4H-1,3-dithiin	C6H8S2	144	4524±119b	9553±76a	n.d.
Miscellaneous							
M1	<800	1-propene	C3H6	42	643±50a	418±62b	312±40c
M2	882	Allyl mercaptan	C3H6S	79	123±18a	111±22a	n.d.
M3	1113	Allyl alcohol	C3H6O	58	120±14a	106±28a	n.d.
M4	1114	2-methyl thiophene	C5H6S	98	n.d.	n.d.	126±9a
M5	1188	2,5-dimethyl thiophene	C6H8S	112	29±3c	48±2b	142±3a
M6	1251	2,4-dimethyl thiophene	C6H8S	112	180±25c	313±62b	816±29a
M7	1364	2,5-dimethyl pyridine	C7H9N	107	102±5b	111±8b	281±8a

¹⁾ Values are shown as peak area/10000 and means $\pm$ SD of triplicates.

²⁾ Significant differences (p<0.05) between roots and leaves of *Allium hookeri* using Duncan's Multiple Comparison Test between the samples having the different letter in a row.

³⁾ n.d., not detected; MF, molecular formula; MW, molecular weight.

Table S3. Total phenolics content and antioxidant activities in hooker chive

Hooker chive		Total phenolics (mg GAE/g) ¹⁾	DPPH (mg/mL) ²⁾	FRAP (593 nm)
Leaves		71.19±3.15a ³⁾	9.59±0.62c	2.36±0.00a
Roots	Cheongju	19.32±0.31c	38.54±1.15a	0.39±0.03c
	Myanmar	22.02±0.18b	35.11±0.37b	0.44±0.96b

¹⁾ Each value is the means ± SD (n=3). GAE, gallic acid equivalent.

²⁾ The antioxidant capacity was expressed IC 50 value, the amounts of samples required to scavenge 50% of DPPH radical.

³⁾ Significant differences (p<0.05) between roots and leaves of hooker chive using Duncan's multiple comparison test between the samples having a different letter in a column

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