Korean J. Plant Res. 27(6):610-621(2014)
http://dx.doi.org/10.7732/kjpr.2014.27.6.610

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Analysis of the Phenolic Content and Antioxidant Activities of Soybean Extracts from Different Regions and Cultivars

Jung-Hwan Nam[1†], Suk-Woo Kang[2†], Su-Young Hong[1], Su-Jeong Kim[1], Young-Ik Jin[1], Hyun-Sam Kim[1], Young-Ho Yoon[1], Jin-Cheol Jeong[1], Cheol-Ho Pan[2], Byung-Hun Um[2], Chu-Won Nho[2] and Hyun-Choong Ok[3]*

[1]Highland Agriculture Research Center, National Institute of Crop Science, RDA, Pyeongchang 232-955, Korea
[2]Functional Food Center, Korea Institute of Science and Technology, Gangneung 210-340, Korea
[3]Research Policy Bureau, RDA, Suwon 441-707, Korea

Abstract - Phenolic compounds such as isoflavones contribute to the antioxidant properties of soybean. This study investigated differences in the phenolic content and antioxidant activity of ten soybean cultivars grown in two different locations in Korea. We observed a wide range of variation in total isoflavone and phenolic content by location and cultivar. The total isoflavone content of cultivars grown at Pyeongchang (sub-highland) was significantly higher than that in cultivars grown at Gangneung (lowland). In contrast, the total phenolic content of cultivars grown at Gangneung was greater than that of cultivars grown at Pyeongchang. The radical scavenging activity of DPPH was similar to that of the total phenolic content rather than that of the total isoflavone content. These results suggest that antioxidant activity of soybean was associated with phenolic compounds rather than isoflavones. To identify the individual antioxidant components, we used an on-line HPLC-ABTS^{+}-based assay system, ESI/MS, and NMR. The results showed that the strongest antioxidant activity was linked to epicatechin.

Key words - Antioxidant activity, Epicatechin, Isoflavone, Phenolic compounds, Soybean

Introduction

Studies suggest that free radicals and reactive oxygen species such as superoxide radicals, hydroxyl radicals, and peroxyl radicals are harmful to human health and that they trigger various conditions, such as cancer, coronary heart diseases, inflammatory disorders, and arteriosclerosis; they are also linked to aging (Ames *et al*., 1993; Nanjo *et al*., 1996; Lee *et al*., 2007; Li *et al*., 2007; Wang *et al*., 2007). Antioxidants may help prevent oxidative damage in the human body (Ames *et al*., 1993; Cos *et al*., 2003). As a result, scientific interest has increased in the levels of antioxidant components in food.

Reports have proposed that the antioxiadant activity of soybean (*Glycine max* L. Merrill) seeds and foods is mainly affected by phenolic compounds such as isoflavone, flavonoids, and phenolic acids (Malencic *et al*., 2007; Riedl *et al*., 2007). Many studies have suggested that the antioxidant activity of soybean seeds and soybean foods was positively correlated with total phenolics and isoflavones (Kim *et al*., 2006; Malencic *et al*., 2008; Devi *et al*., 2009). Several studies exhibit that genetic variability affects seed phenolic content of soybean cultivars, and phenolic content variations can also be affected by environmental conditions (Malencic *et al*., 2007; Hoeck *et al*., 2000; Lee *et al*., 2003). A number of studies have recently focused on the bioactivity of phenolic compounds, especially isoflavone in terms of its antioxidant and anticancer properties (Lamartiniere, 2000; Mezei *et al*., 2003).

The majority of studies conducted thus far have focused on the antioxidant activity and phenolic compounds of soybean. Few studies have attempted to identify the major components that contribute to the antioxidant activity of soybean. As soybean contains many different types of phenolic compounds (Kim *et al*., 2004; Lee *et al*., 2008), it is feasible that the composition and content of the compounds will influence the level of

*Corresponding author. E-mail : hycok@korea.kr
† These authors contributed equally to this work.

antioxidant activity.

The antioxidant activity and phenolic composition of soybeans may differ according to their genotype or environmental condition. To investigate the beneficial condition for higher antioxidant content, it is important to examine the different soybean cultivars in different environment. Profiling soybean antioxidant activity, phenolic content and isoflavone content can help elucidate what is the major factor for antioxidant activity of soybean and lead to soy products with greater health benefits. In this study, we evaluated the isoflavone, total phenolic content and antioxidant activity of ten soybean cultivars produced from different regions to identify the major antioxidant components of Korean soybean cultivars.

Materials and Methods

Soybean seed and plant cultivation

Ten popular soybean cultivars were grown at two regions; Gangneung (GN) (lowland, 20 m altitude) and Pyeongchang (PC) (sub-highland, 600 m altitude) in Korea in 2008. The cultivars differed in seed weight, seed coat color, and cotyledon color (Table 1). The soil type was a silt clay loam at all regions.

Each plot consisted of ten rows (5 m long and 0.6 m between rows). The experiment consisted of a completely randomized design with three replicates. The planting arrangement was 60 ㎝ × 15 ㎝ per plot, and the plants were thinned to make a uniform density 20 days after planting. The plots were hand weeded during June and July and appropriate pesticides were applied to control diseases and insects. Fertilizers were applied prior to plowing at the recommended rates of 8, 8 and 12 ㎏ per 1,000 ㎡ for N, P_2O_5 and K_2O, respectively. All other soybean cultivation procedures followed the standard methods used in Korea (Ryu *et al*., 2001) Soybean seeds were harvested from each replicate at each region. The harvested seed samples were freeze dried, ground, and stored at -80℃ prior to analysis of phytochemical and antioxidant activity.

Chemicals

The three aglycon standards of isoflaovne (daidzein, genistein, and glycitein), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO, USA). Dimethyl sulfoxide-d_6 was purchased from Cambridge Isotope Laboratory. All HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All of the chemicals and solvents were analytical grade or HPLC grade.

Isoflavone analysis

Following acid hydrolysis of the endogenous 12 isoflavones to their aglycon forms, the concentraions of daidzein, genistein, and glycitein (summed to obtain total isoflavone) were

Table 1. Characteristics of the soybean cultivars used in this study

Cultivars	Symbol	Weight of 100 grains (g)		Seed coat color	Cotyledon color	Growth type
		GN[z]	PC[y]			
Daepoongkong	DP	25.1 ± 0.8[x]	21.9 ± 0.3	Yellow	Yellow	Determinate
Taekwangkong	TK	29.4 ± 0.2	22.0 ± 0.4	Yellow	Yellow	Determinate
Eunhakong	EH	15.9 ± 0.3	14.0 ± 0.2	Yellow	Yellow	Determinate
Poongsannamulkong	PS	12.0 ± 0.4	10.4 ± 0.1	Yellow	Yellow	Determinate
Geomjeongkong4	GJ	34.5 ± 0.3	30.4 ± 0.2	Black	Yellow	Determinate
Ilpoomgeomjeongkong2	IP	36.0 ± 0.3	34.5 ± 1.0	Black	Yellow	Determinate
Cheongjakong3	CJ	36.7 ± 0.1	33.4 ± 0.5	Black	Green	Determinate
Heukmikong	HM	29.3 ± 1.4	28.7 ± 0.5	Black	Green	Determinate
Socheongkong	SC	18.5 ± 0.8	13.8 ± 0.1	Black	Green	Determinate
Jwinunyikong	JN	10.5 ± 0.1	9.2 ± 0.1	Black	Green	Determinate

[z]Gangneung: 37°N, 128°E, 20 m altitude.
[y]Pyeongchang: 37°N, 128°E, 600 m altitude.
[x]mean ± standard error.

determined using a modified version of the HPLC method of Franke *et al*. (1994).

Soybean seeds were powdered using a mill with a ㎜ sieve. Two grams of dry ground sample were suspended in 10 ㎖ of 1 N HCl and heated for 2 h at 100℃. The extracts were allowed to cool, mixed with 30 ㎖ of methanol, and stirred for 24 h at 40℃. The solution was then filtered through Whatman No. 42 filter paper, and the filter was rinsed three times with methanol. The filtrate was transferred to a 50 ㎖ volumetric flask. The sample was filtered through a 0.45 ㎛ PTFE filter (Pall, Ann Arbor, MI, USA, and 1 ㎖ of the filtrate was transferred to sample vials for HPLC analysis.

The concentrations of individual hydrolyzed daidzein, genistein, and glycitein were determined using a HPLC system (Waters, Alliance system, Milford, MA, USA) consisting of a 2695 Separation Module pump and a 2996 Photodiode Array Detector. Twenty microliters of sample were injected into a Phenomenex ODS Hypersil C18 column (5 ㎛ particle size, 250 ㎜ × 4.6 ㎜, Phenomenex, Torrance, CA, USA). A linear HPLC gradient was used; solvent A was 0.1% aq. acetic acid in nanopure water, and solvent B was 0.1% aq. acetic acid in acetonitrile. Following the injection of the sample, solvent B was increased from 15 to 35% for 50 min and then maintained at 35% for 10 min. The solvent flow rate was 1 mL/min. The wavelength of the UV detector was set at 254 ㎚. The MassLynx software associated with the Waters HPLC instrument was used to generate linear calibration curves based on peak areas for the three isoflavone standards run with each sample batch. Peak areas were generally used to analyze the isoflavone concentrations quantitatively. The term 'total isoflavone' in this study refers to the sum of daidzein, genitein, and glycitein.

Determination of total phenolic compounds

Total phenolic compounds of soybean extracts were determined according to the previous method (Kozlowska *et al*., 1983; Zielinski and Kozlowska, 2000) with minor modifications. Two grams of ground soybean seed were mixed with 20 mL of 100% HPLC grade methanol in a 50 ㎖ screw-top flask, stirred for 24 h at 30℃, and filtered through a Whatman No. 42 filter paper. The filtrate was dried in a vacuum rotary evaporator (Eyela, Tokyo, Japan) below 30℃. Dried sample extracts were redissolved in 1 ㎖ of 100% methanol. A 0.25 ㎖ methanol extract of soybean was mixed with 4 ㎖ of distilled water and 0.25 ㎖ of Folin-Ciocalteu reagent, vortexed for 30 s, and left to stand for 5 min. The mixture was then added to 0.5 ㎖ of saturated sodium carbonate and allowed to stand for 30 min in the dark.

The absorbance was measured by Lambda 35 UV/vis spectrometer (Perkin-Elmer, Waltham, MA, USA) at 725 ㎚ against distilled water as blank. Each sample was analyzed in triplicate, and the results were expressed as gallic acid equivalents (milligrams of gallic acid equivalents per gram of sample) through the calibration curve of gallic acid.

Free radical scavenging activity

The free radical scavenging activities of soybean cultivars were determined using the DPPH scavenging method (Ozcelik *et al*., 2003). DPPH was dissolved in 100% methanol to a concentration of 0.5 mM. A total of 3.75 ㎖ of 0.5 mM DPPH was then mixed with 0.25 ㎖ of methanol extract as prepared above. The reaction mixture was maintained in the dark at room temperature for 30 min. The absorbance at 517 ㎚ was then recorded by Lambda 35 UV/vis spectrometer (Perkin-Elmer, Waltham, MA, USA). The free radical scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [A_a - (A_b - A_c)] / A_a \times 100,$$

where A_a is the absorbance of the incubation solution of DPPH without the sample solution, A_b is the absorbance of the incubation mixture containing both sample solution and DPPH, and A_c is the absorbance of the blank solution without DPPH.

On-line detection of radical scavenging activity

The radical scavenging activity of the soybean seed was determined using the on-line $ABTS^+$ assay according to the method of Stewart *et al*. (2005) with some modification. A 2 mM $ABTS^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated overnight in the dark at room temperature, to allow for the stabilization of the radicals. The $ABTS^+$ reagent was prepared by diluting the stock 8-fold in methanol. Ten milligrams of each sample extract was dissolved in 1 ㎖ of methanol and filtered in a 0.2 ㎛ Acrodisc syringe

filter (Pall, Lawrence, KS, USA), and 10 μl of this solution was injected to an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with binary pumps, a diode array detection (DAD), a UV/VIS detector, and an additional reagent pump. The analytical column was a Hydrosphere-C18 column (5 μm particle size, 150 mm × 4.6 mm, YMC Co., Kyoto, Japan). The mobile phase consisted of acetonitrile (solvent A) and water (solvent B). A linear gradient (from 5 to 95% A for 60 min and 95% A for 10 min) was used. The chromatographic profile was recorded at 210 and 320 nm. The flow rate was 0.5 ml/min. The analyses were performed at 40℃. The HPLC eluent from the DAD arrived at a 'T' piece, where the $ABTS^+$ was added. The $ABTS^+$ flow rate was 0.25 ml/min delivered by an additional Agilent 1200 pump. After mixing through a 1-ml loop maintained at 40℃, the absorbance was measured at 734 nm by a UV/VIS detector. Finally, the data were analyzed using ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

Identification of compounds by HPLC-ESI/MS and NMR

The compounds were identified by a Varian 1200L LC-MS system (Palo Alto, CA, USA) with an electrospray source. The Varian MS workstation software (version 6.3) was used for data acquisition and processing. The HPLC conditions were the same as described above for the on-line detection of radical scavenging activity. The mass spectrometer conditions were as follows: positive ion mode, mass range = m/z 150-1,000, needle = 5,000 V, shield = 600 V, nebulizing gas pressure (N_2) = 60 psi, drying gas (N_2) flow rate = 20 psi, and drying temperature = 300℃.

Four major peaks were isolated by JASCO semi-preparative HPLC system (JASCO Co. Tokyo, Japan), which is equipped with PU-2086 Plus pumps, an MD-2015 PDA detector, and an MX2080-31 solvent mixing module. Separations were performed on a Hydrosphere-C18 column (5 μm particle size, 250 mm × 10 mm, YMC Co., Kyoto, Japan) with acetonitrile-water (5% acetonitrile at 0 min, 95% acetonitrile at 50 min) at a flow rate of 10 ml/min.

^{1}H- and ^{13}C-NMR data were acquired using a 500 MHz Varian NMR system (Varian, Palo Alto, CA, USA) equipped with a cold probe. Data were processed by Vnmrj software.

Tryptophan (1) ESI-MS *m/z*: 205 $[M+H]^+$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 2.92 (1H, dd, *J*=15.1, 9.8 Hz, H-10a), 3.29 (1H, dd, *J*=15.1, 3.8 Hz, H-10b), 3.39 (1H, dd, *J*=9.1, 3.8 Hz, H-11), 6.98 (1H, t, *J*=7.7 Hz, H-5), 7.06 (1H, t, *J*=7.7 Hz, H-6), 7.18 (1H, d, *J*=2.1 Hz, H-2), 7.34 (1H, d, *J*=7.8 Hz, H-7),7.37 (2H, brs, NH_2) 7.55 (1H, d, *J*=7.8 Hz, H-4), and 10.9 (1H, brs, NH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 27.7 (C-10), 55.3 (C-11), 109.7 (C-3), 111.7 (C-7), 118.7 (C-5), 118.8 (C-4), 121.4 (C-6), 124.4 (C-2), 127.7 (C-9), 136.8 (C-8), and 170.8 (C-12).

Epicatechin (2) ESI-MS *m/z*: 291 $[M+H]^+$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 2.46 (1H, dd, *J* = 16.4, 3.1 Hz, H-4a), 2.67 (1H, dd, *J* = 16.4, 4.5 Hz, H-4b), 3.99 (1H, ddd, *J* = 4.5, 3.1, 1.1 Hz, H-3). 4.68 (1H, brs, OH-3), 4.72 (1H, d, *J* = 1.1 Hz H-2), 5.71 (1H, d, *J* = 2.3 Hz, H-8), 5.89 (1H, d, *J* = 2.3 Hz, H-6), 6.64 (1H, dd, *J* = 8.2, 1.7 Hz, H-6′), 6.66 (1H, d, *J* = 8.1 Hz, H-5′), 6.88 (1H, d, *J* = 1.7 Hz, H-2′), and 8.80-9.12 (4H, brs, OH-5/7/3′/4′). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 28.7 (C-4), 65.3 (C-3), 78.5 (C-2), 94.5 (C-8), 95.5 (C-6), 98.9 (C-10), 115.2 (C-2′), 115.4 (C-5′), 118.4 (C-6′), 131.0 (C-1′), 144.9 (C-3′), 145.0 (C-4′), 156.2 (C-9), 156.7 (C-7), and 157.0 (C-9).

Daidzin (3) ESI-MS *m/z*: 417 $[M+H]^+$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 3.18 (1H, m, H-4″), 3.30 (2H, m, H-2″/3″), 3.45 (2H, m, H-5″/6″a), 3.71 (1H, m, H-6″b), 4.64 (1H, brs, OH-6″), 5.11 (1H, d, *J* = 7.4 Hz, H-1″), 5.13 (1H, brs, OH-4″), 5.19 (1H, brs, OH-3″), 5.48 (1H, brs, OH-2″), 6.82 (2H, m, H-3′/5′), 7.14 (1H, dd, *J* = 8.9, 2.3 Hz, H-6), 7.24 (1H, d, *J* = 2.3 Hz, H-8), 7.41 (2H, m, H-2′/6′), 8.05 (1H, d, *J* = 8.9 Hz, H-5), 8.40 (1H, s, *J* = 8.1 Hz, H-2), and 9.59 (1H, brs, OH-4′). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 61.1 (C-6″), 70.0 (C-4″), 73.6 (C-2″), 76.9 (C-3″), 77.7 (C-5″), 100.4 (C-1″), 103.8 (C-8), 115.4 (C-3′/5′), 116.0 (C-6), 118.9 (C-10), 122.7 (C-1′), 124.1 (C-3), 127.4 (C-5), 130.5 (C-2′/6′), 153.8 (C-2), 157.5 (C-4′), 157.7 (C-9), 161.8 (C-7), and 175.2 (C-4).

Genistin (4) ESI-MS *m/z*: 433 $[M+H]^+$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 3.16 (1H, m, H-4″), 3.29 (2H, m, H-2″/3″), 3.45 (2H, m, H-5″/6″a), 3.70 (1H, m, H-6″b), 4.63 (1H, m, OH-6″), 5.07 (1H, d, *J* = 7.5 Hz, H-1″), 5.12 (1H, d, *J* = 4.3 Hz, OH-4″), 5.21 (1H, brs, OH-3″), 5.45 (1H, d, *J* = 4.4 Hz, OH-2″), 6.47 (1H, d, *J* = 2.0 Hz, H-6), 6.72 (1H, d, *J* = 2.0 Hz,

Table 2. Mean and significance of the mean squares for phytochemical content and bioactivity of the ten soybean cultivars grown at two regions in Korea

		Total isoflavone contents (μg/g)						Total phenolics content[y]		DPPH radical scavenging activity (%)	
		Daidzein		Genistein		Total[z]					
Region	GN	665	b[x]	615	b	1,361	b	4.2	a	45.5	a
	PC	708	a	655	a	1,444	a	3.3	b	39.3	b
Cultivar	DP	973	a	1,113	a	2,245	a	3.7	b	34.7	e
	TK	394	g	332	g	781	g	2.3	c	32.5	e
	EH	993	a	793	b	1,919	b	3.4	b	34.4	e
	PS	735	c	610	d	1,413	d	3.1	bc	33.1	e
	GJ	838	b	657	c	1,564	c	4.0	b	40.9	d
	IP	639	d	606	d	1,334	d	3.7	b	50.0	b
	CJ	536	f	617	cd	1,190	e	3.9	b	50.6	b
	HM	593	e	513	f	1,162	e	3.8	b	43.2	cd
	SC	738	c	574	de	1,394	d	3.9	b	45.3	c
	JN	425	g	532	ef	1,026	f	5.6	a	59.5	a
Location		**[w]		**		**		**		**	
Cultivar		**		**		**		**		**	
L X C		**		**		**		NS[v]		**	

[z]Total = daidzein + genistein + glycitein.
[y]mg of gallic acid equivalent per g of soybean.
[x]Data in the same column with different letters are significantly different at $p < 0.05$.
[w]*, **Mean squares significant at the 0.05 and the 0.01 probability levels, respectively.
[v]Mean squares not significant at the 0.05 probability level.

H-8), 6.83 (2H, m, H-3′/5′), 7.40 (2H, m, H-2′/6′), 8.43 (1H, s, J = 8.1 Hz, H-2), 9.68 (1H, brs, OH-4′), and 12.95 (1H, brs, OH-5). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 61.0 (C-6″), 70.0 (C-4″), 73.5 (C-2″), 76.9 (C-3″), 77.7 (C-5″), 94.9 (C-8), 100.0 (C-6), 100.3 (C-1″), 106.9 (C-10), 115.5 (C-3′/5′), 121.4 (C-3), 123.0 (C-1′), 130.6 (C-2′/6′), 155.2 (C-2), 157.7 (C-9), 158.0 (C-4′), 161.8 (C-5), 163.5 (C-7), and 180.8 (C-4).

Statistical analysis

The soybean was cultivated using a completely randomized design and replicated three times at two locations. The analysis of the isoflavones by HPLC was repeated four times with three extracts in each cultivar. Free radical scavenging activity and total phenolic compounds were measured in triplicates. The data were analyzed statistically by analysis of variance (ANOVA). The Duncan's multiple range tests (probability level 0.05) were used to analyze the difference between the means of the samples using the SAS program (Table 1 and Table 2).

Results and Discussion

Isoflavones

In soybeans, isoflavones are present both as aglycones and as glycosides (β-glycosides, acetyl glycosides, and malonyl glycosides), the latter being the predominant form (Lee *et al*., 2003; Lee *et al*., 2004; Kim *et al*., 2005). The biosynthetic pathway of isoflavones that leads to the accumulation of individual isoflavone compounds does not differ a great deal, although the total isoflavone content is influenced by the environment, cultivar, and their interaction (Sakthivelu *et al*., 2008). For this reason, we selected to measure isoflavone aglycone content following acid hydrolysis to investigate the impact of cultivar and planting region on the variation in the isoflavone content of soybean seeds.

The isoflavone content of the ten soybean cultivars grown at the two regions in Korea are shown in Fig. 1 and Table 2. The range of total isoflavone was 720 to 2,285 μg/g, which is similar to that reported in previous studies conducted in

Korea (So *et al*., 2001; Lee *et al*., 2002). At GN (altitude 20 m), the total isoflavone content ranged from 720 to 2,205 ㎍/g and the average total isoflavone content was 1,361 ㎍/g. At PC (altitude 600 m), the total isoflavone content ranged from 841 to 2,285 ㎍/g and the average total isoflavone content was 1,444 ㎍/g. Therefore, the total average concentration of isoflavones in soybean cultivated at PC was significantly higher than at GN. Among the cultivars examined in this study, DP showed the highest total isoflavone content at both growing regions and TK exhibited the lowest concentrations at both regions. Together, there was a wide range of variation in the total isofalvone content of the cultivars. Most of the cultivars grown at the sub-highland PC site contained higher isoflavone content than those grown at the lowland GN site. PS and GJ contained a higher concentration of isoflavones when they were cultivated at GN. These results indicate that the cultivar × region interaction had a significant impact on the total isoflavone content (Table 2).

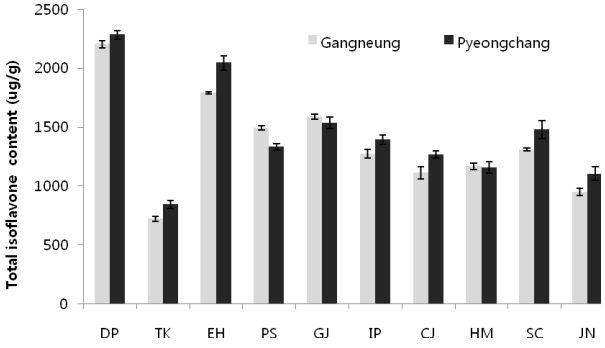

Fig. 1. Isoflavone content of the ten soybean cultivars grown at two different locations in Korea. Each bar indicates the standard deviation values.

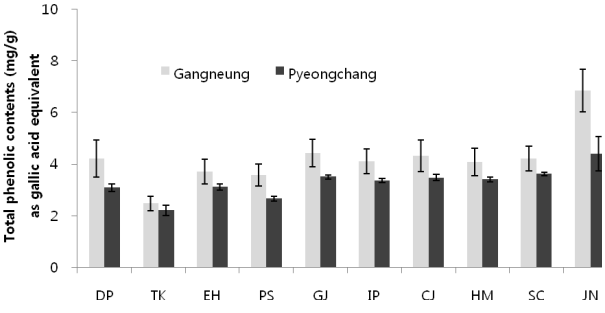

Fig. 2. Total phenolic content of the ten soybean cultivars grown at two different locations in Korea. Each bar indicates the standard error values.

Planting location and genotype may explain the variation in total isoflavone content. Hoeck *et al*. (2000) and Riedl *et al*. (2007) previously confirmed the influence of planting location and cultivar on the total isoflavone content at four sites in Ohio and at eight sites in Iowa, respectively. Another study reported that low growing temperature during the first few weeks after seeding promote isoflavone biosynthesis in soybean (Tsukamoto *et al*., 1995). In common with previous studies, we found that isoflavone concentrations are influenced by various environmental factors, such as cultivation site and temperature, as well as by genetically controlled factors, such as cultivar, seed weight, and seed color (Hoeck *et al*., 2000; Lee *et al*., 2003).

Total phenolic compounds

We found that the total phenolic content ranged from 2.2 to 6.9 ㎍/g (Fig. 2). The total phenolic content of the cultivars grown at GN was 2.5 to 6.9 ㎍/g, with an average of 4.2 ㎍/g. At PC, the total phenolic content ranged from 2.5 to 4.4 ㎍/g, with an average of 3.3 ㎍/g (Table 2).

All of the cultivars grown at GN had greater total phenolic content than those grown at PC. Among the cultivars studied, JN had the highest concentration of total phenolic content at both growing locations. In particular, JN grown at GN had much higher total phenolic content (6.9 ㎎/g) than any of the other samples. TK exhibited the lowest total phenolic content of all the cultivars at both sites. The other eight cultivars exhibited little variation in total phenolic content, and there was no significant difference between these cultivars in total phenolic contents (Table 2). The trend in total phenolic content contrasted with our findings for isoflavone concentrations, which were higher in cultivars grown at PC where was also a significant difference between the cultivars in total isoflavone concentrations.

As isoflavones are phenolics and likely a major contributor to the total phenolic content, it is natural to assume that the total phenolic content is closely associated with the total isoflavone content. Some researchers have reported that the total phenolic content is positively correlated with the total isoflavone concentration (Riedl *et al*., 2007; Sakthivelu *et al*., 2008; Devi *et al*., 2009). However, we did not find any such correlation between total phenolics and isoflavones. It is

suggested more phenolic compounds except isoflavone are included in soybeans cultivated in GN. The isoflavone content of soybean cultivars used in this study was higher at PC, whereas the total phenolic content was greater at GN. Among the cultivars studied, the highest isoflavone content was observed in DP; while the highest total phenolic content was detected in JN.

Previous studies have reported that soybean contains several phenolic acids, such as syringic, ferulic, sinapic, coumaric, hydroxybenzoic, caffeic, and chlorogenic acids (Kozlowska *et al*., 1983) and that total phenolic compounds are highly positively correlated with hydroxybenzoic acid, such as gentisic acid and salicylic acid (Kim *et al*., 2004). Lee *et al*. (2008) reported that soybean cultivars grown in Korea contain 28 phenolic compounds including eleven hydroxybenzoic acids, seven hydroxycinnamic acids, eight flavonoids, and two others. Considering that several phenolic compounds are present in soybean, it is feasible that the total phenolic content is not always correlated with the total isoflavone content. Further studies are needed to investigate the relationship between total phenolics and isoflavones and to identify all of the phenolic compounds present in soybean.

Antioxidant activity

The antioxidant activities of the ten soybean cultivars grown at the two locations are shown in Fig. 3 and Table 2 as a percentage of the radical scavenging activity of DPPH. There were significant differences in the free radical scavenging activities of the cultivars ($P < 0.05$). JN cultivated at GN exhibited the highest free radical scavenging activity (67.8%) whereas PS cultivated at PC had the lowest (30.6%). The average free radical scavenging activities of the ten soybean cultivars grown at GN and at PC were 45.5 and 39.3%, respectively. There was a significant difference in DPPH radical scavenging activity between the two sites. Our findings are similar to those of Lee *et al*. (2002), who concluded that the free radical scavenging activities of soybean extracts are influenced by genetic and environmental variations.

Overall, the trend in DPPH radical scavenging activity was similar to that of total phenolic content rather than that of total isoflavone content. Cultivars with the lowest total phenolic content, such as TK and PS exhibited only about half of the

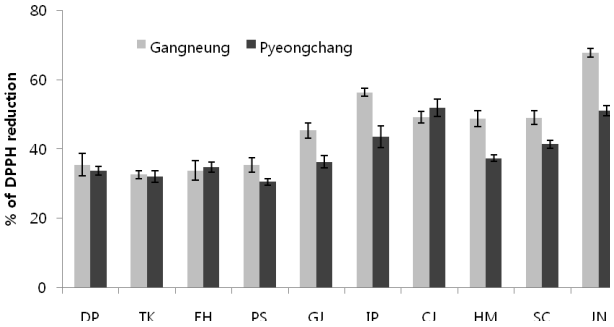

Fig. 3. Free radical scavenging activity of the ten soybean cultivars grown at two different locations in Korea. Each bar indicates the standard error values.

antioxidant activity of the phenolic-rich cultivar, JN. Despite having low total isoflavone content, JN, CJ, and IP showed high levels of DPPH radical scavenging activity.

Both isoflavone aglycones and glycosides were present in soybean, with glycosides as the predominant isoflavone form (Lee *et al*., 2003; Lee *et al*., 2004; Kim *et al*., 2005). Previous research suggested that glucose linkage to aglycone reduced the antioxidant activities of isoflavones approximately 50 to 100 times (Naim *et al*., 1976). Moreover, Cos *et al*. (2003) reported that isoflavones were less potent in a DPPH assay compared with certain phenolic acids present in soybean. Lee *et al*. (2005) suggested that many isoflavones have low scavenging potency for DPPH free radicals, with scavenging effects only half that of α-tocopherol and one-third that of epicatechin. Therefore, it is possible that phenolic acids or some other compounds present in soybean seeds were responsible for much of the DPPH radical scavenging activity detected in this study.

On-line HPLC-ABTS$^+$ radical scavenging activity and the identification of compounds by HPLC-ESI/MS and NMR

Many methods, such as ABTS and DPPH assay have been used to evaluate the free radical scavenging capacity of compounds. However, these methods cannot determine the active components in a mixture. Recently, a technique has been developed that measures the radical scavenging activity of individual compounds on-line when they elute from the HPLC column (Koleva *et al*., 2001; Lee *et al*., 2007). This technique allow for rapid and selective detection of radical scavenging compounds in the presence of many other inactive

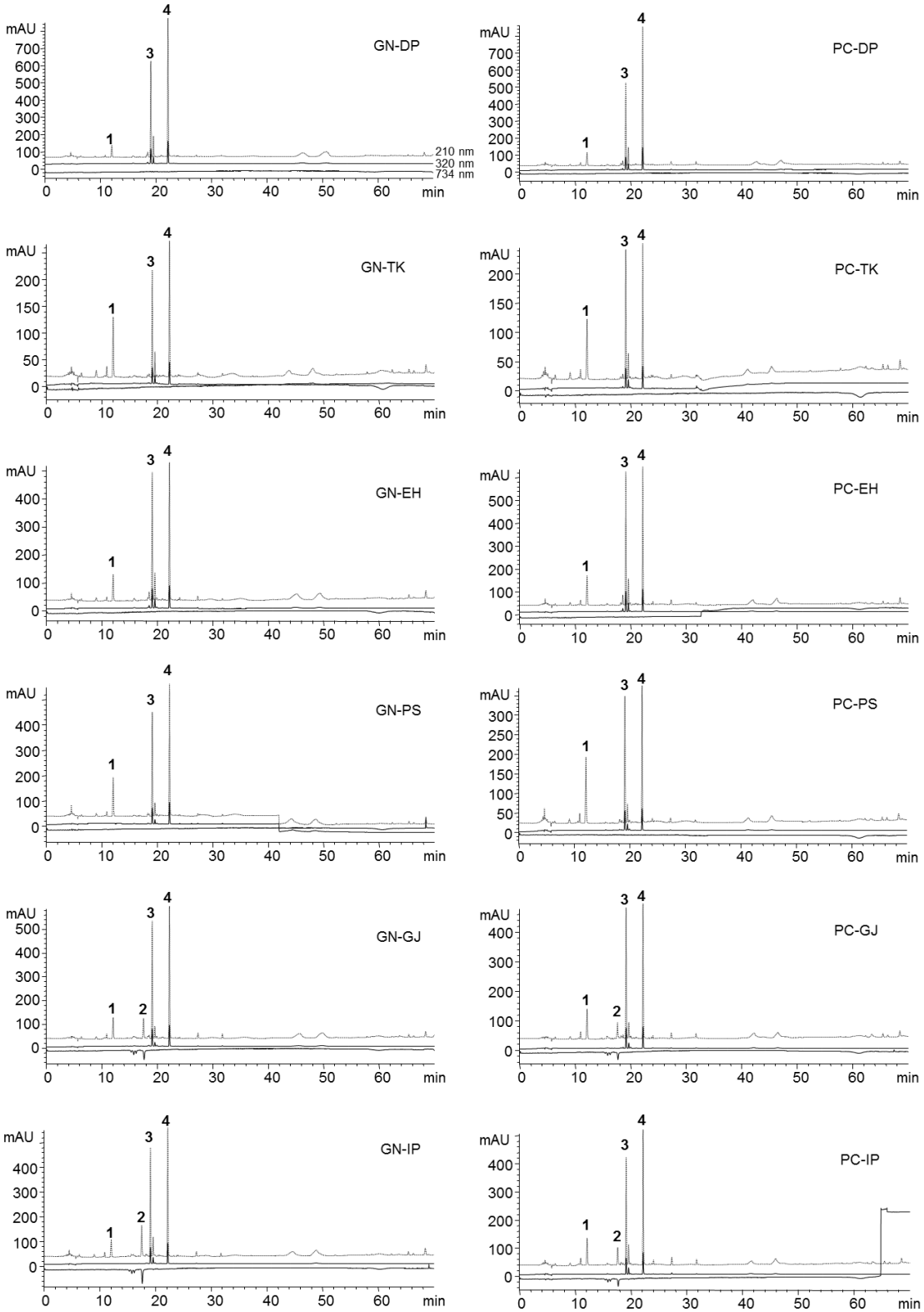

Fig. 4. On-line HPLC-ABTS+ analysis of soybean extract. Ten milliliters were analyzed by gradient reverse-phase HPLC with a DAD at 210 ㎚ (positive trace, dotted line) and 320 ㎚ (positive trace, solid line) prior to reaction with the $ABTS^{+}$ radical and the analysis of antioxidant potential at 734 ㎚ (negative trace). (tryptophan **(1)**, epicatechin **(2)**, daidzin **(3)**, and genistin **(4)**).

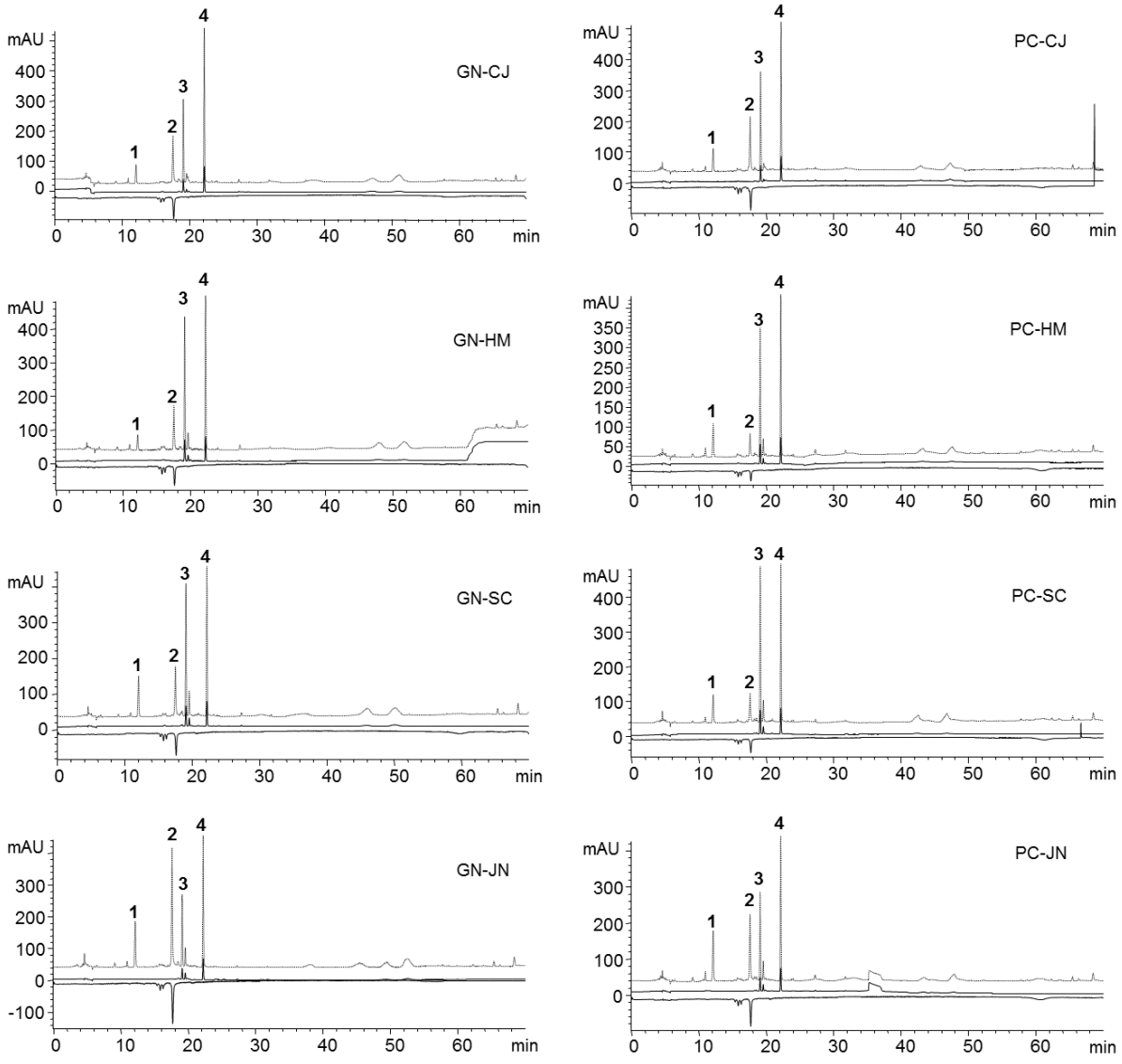

Fig. 4. Continued.

constituents with minimal sample preparation. We used HPLC coupled with the on-line $ABTS^{+}$-based assay system and HPLC-ESI/MS to rapidly identify radical scavenging compounds in soybean extract.

An example of the antioxidant analysis of soybean extract is shown in Fig. 4. The upper part (positive) of the figure shows the UV chromatogram as measured at 210 (dotted) and 320 ㎚ (solid line). The down part (negative) of the figure shows the corresponding $ABTS^{+}$ radical scavenging activities of the same compounds. The chromatographic analyses of the soybean extract at 210 and 320 ㎚ revealed the existence of four major compounds, which were identified as tryptophan (**1**), epicatechin (**2**), daidzin (**3**), and genistin (**4**) by LC-MS and NMR (Fig. 5). Among the four compounds, epicatechin exhibited the strongest antioxidant activity.

The UV spectrum of peak 2 showed maximum absorbance at 225 and 277 ㎚. The mass analysis of peak 2 showed a $[M+H]^{+}$ ion at m/z 291 for ESI-MS in the positive ion mode. Based on the UV and MS spectral data and previously reported literature (Zeeb *et al*., 2000; Qian *et al*., 2008; Vasco *et al*., 2009), peak 2 was identified as epicatechin. The NMR data showed that coupling between H-2 and H-3 was negligible (J = 1.1 Hz), thereby confirming that peak 2 was epicatechin. If there is a *trans* relationship between C-2 and C-3, large coupling

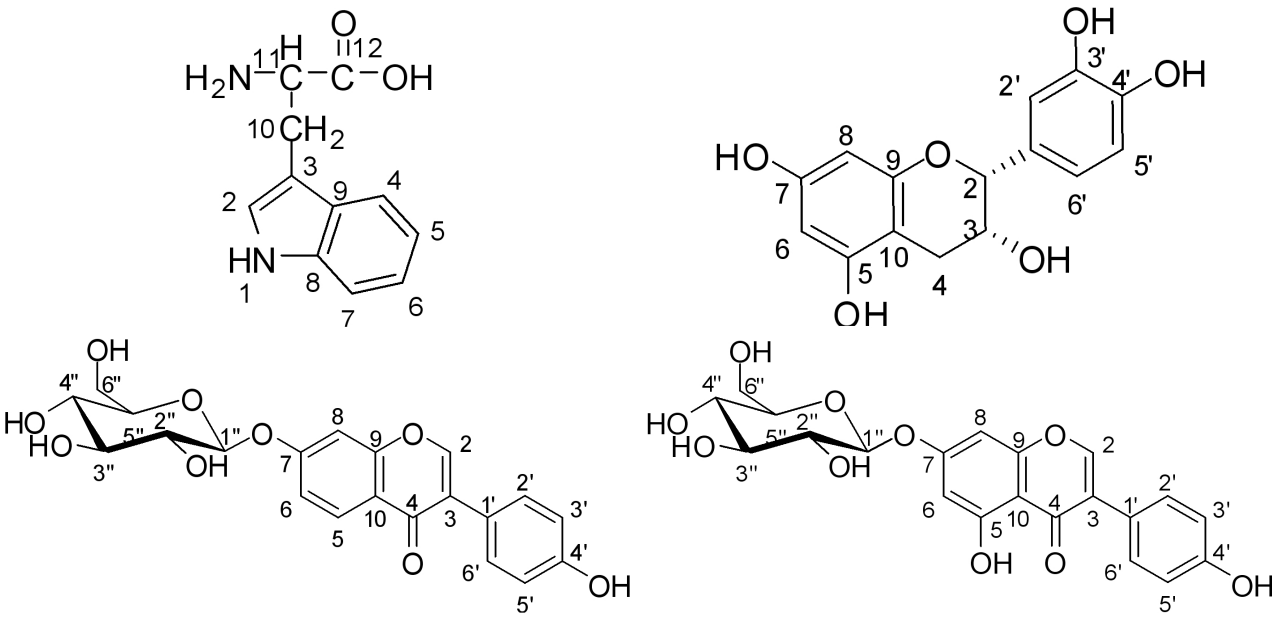

Fig. 5. Structures of the four major compounds present in the soybean extract.

(J = 8.0 Hz) should be observed (Hsue-Fen *et al*., 1993).

Catechin and epicatechin are flavonoids that are widespread in many edible plants such as green tea. Numerous studies have addressed the antioxidative activities of these flavonoids in scavenging free radicals, chelating metal ions, preventing the activation of redox-sentitive transcription factors, and inhibiting prooxidant enzymes (Medina *et al*., 2007; Stangl *et al*., 2006). In particular, epicatechin was reported to exhibit antioxidant activity three times higher than that of many isoflavones (Lee *et al*., 2005). Lee *et al*. (2008) reported that catechin is present in soybean seed and Cho *et al*. (2009) reported that both catechin and epicatechin are present in soybean-fermented food. However, the antioxidant activity of epicatechin in soybean has not been reported previously. The antioxidant detection system used in this study clearly indicates for the first time that epicatechin makes a major contribution to the total antioxidant activity of soybean seeds.

In this study, we showed total isoflavones, total phenolics, and antioxidant activity of soybeans differed by locations (altitude) or cultivars. The antioxidant activity could be largely linked to total phenolics rather than isoflavones. Correlations among phenolic content, location and genotype found in this study can help distinguish variables associated with antioxidant activity of soybeans. However, more environmental variations such as temperature, precipitation, humidity and sunshine need to be investigated.

In addition, the on-line HPLC-ABTS^{+} method was applied successfully to evaluate the ABTS-radical scavenging activity of each peak concurrently. We demonstrated that epicatechin is the most potent antioxidant constituent comprised in those soybean seeds.

Acknowledgment

This study was supported by an intramural grant (2Z03850) from Korea Institute of Science and Technology, Gangneung Institute.

References

Ames, B.N., M.K. Shigenaga and T.M. Hagen. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA. 90:7915-7922.

Cos, P., N. Hermans, M. Calomme, L. Maes, T. De Bruyne, L. Pieters, A.J. Viletinck and D.V. Berghe. 2003. Comparative study of eight well-known polyphenolic antioxidants. J. Pharm Pharmacol. 55:1291-1297.

Cho, K.M., S.Y. Hong, R.K. Math, J.H. Lee, D.M. Kambiranda, J.M. Kim, S.M.A. Islam, M.G. Yun, J.J. Cho, W.J. Lim and H.D. Yun. 2009. Biotransformation of phenolics (isoflavones,

flavanols and phenolic acids) during the fermentation of cheonggukjang by *Bacillus pumilus* HY1. Food Chem. 114:413-419.

Devi, M.K.A., M. Gondi, G. Sakthivelu, P. Giridhar, T. Rajasekaran and G.A. Ravishankar. 2009. Functional attributes of soybean seeds and products, with reference to isoflavone content and antioxidant activity. Food Chem. 114:771-776.

Franke, A.A., L.J. Custer, C.M. Cerna and K.K. Narala. 1994. Quantitation of phytoestrogens in legumes by HPLC. J. Agric. Food Chem. 42:1905-1913.

Hoeck, J.A., W.R. Fehr, P.A. Murphy and G.A. Welke. 2000. Influence of genotype and environment on isoflavone contents of soybean. Crop Sci. 40:48-51.

Hsue-Fen, C., T. Tanaka, G.I. Nonaka, T. Fujioka and K. Mihashi. 1993. Phenylpropanoid-substituted catechins from *Castanopsis hystrix* and structure revision of cinchonains. Phytochemistry 33:183-187.

Kim, S.H., H.K. Song, J.K. Ahn, J.T. Kim, S.J. Hahn and I.M. Chung. 2004. Changes of phenol compounds according to storing years in soybean. Kor. J. Crop Sci. 49:82-88 (in Korean).

Kim, S.L., H.Y. Chi, J.T. Kim, Y.H. Lee, N.K. Park, J.R. Son and S.J. Kim. 2006. Isoflavone content and its relationship with other seed quality traits of soybean cultivars collected in South Korea. Kor. J. Crop Sci. 51:81-88 (in Korean).

Kim, S.L., H.B. Kim, H.Y. Chi, N.K. Park, J.R. Son, H.T. Yun and S.J. Kim. 2005. Variation of anthocyanins and isoflavones between yellow-cotyledon and green-cotyledon seeds of black soybean. Food Sci. Biotechnol. 14:778-782.

Koleva, I.I., H.A.G. Niederlander and T.A. van Beek. 2001. Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates. Anal. Chem. 73:3373-3381.

Kozlowska, H., R. Zadernowski and F.W. Sosulsk. 1983. Phenolic-acids in oilseed flours. Nahrung-Food 27:449-453.

Lamartiniere, C.A. 2000. Protection against breast cancer with genistein: a component of soy. Am. J. Clin. Nutr. 71:1705-1707.

Lee, C.H., L. Yang, J.Z. Xu, S.Y.V. Yeung, Y. Huang and Z.Y. Chen. 2005. Relative antioxidant activity of soybean isoflavones and their glycosides. Food Chem. 90:735-741.

Lee, J.H., I.Y. Baek, N.S. Kang, J.M. Ko, H.T. Kim, C.S. Jung, K.Y. Park, Y.S. Ahn, D.Y. Suh and T.J. Ha. 2007. Identification of phenolic compounds and antioxidant effects from the exudate of germinating peanut (*Arachis hypogaea*). Food Sci. Biotechnol. 16:29-36.

Lee, J.H., M. Renita, R.J. Fioritto, S.K. St Martin, S.J. Schwartz and Y. Vodovotz. 2004. Isoflavone characterization and antioxidant activity of Ohio soybeans. J. Agric. Food Chem. 52:2647-2651.

Lee, S.J., J.K. Ahn, S.H. Kim, J.T. Kim, S.J. Han, M.Y. Jung and I.M. Chung. 2003. Variation in isoflavone of soybean cultivars with location and storage duration. J. Agric. Food Chem. 51:3382-3389.

Lee, S.J., I.M. Chung, J.K. Ahn, S.K. Lee, S.H. Kim and N.H. Yoo. 2002. Variation in antioxidative activity of soybean (*Glycine max* L.,) varieties with crop year and duration of storage time. Food Sci. Biotechnol. 11:649-653.

Lee, S.J., J.J. Kim, H.I. Moon, J.K. Ahn, S.C. Chun, W.S. Jung, O.K. Lee and I.M. Chung. 2008. Analysis of isoflavones and phenolic compounds in Korean soybean [*Glycine max* (L.) Merrill] seeds of different seed weights. J. Agric. Food Chem. 56:2751-2758.

Lee, S.J., W.K. Yan, J.K. Ahn and I.M. Chung. 2003. Effects of year, site, genotype and their interactions on various soybean isoflavones. Field Crops Res. 81:181-192.

Li, S.Y., Y. Yu and S.P. Li. 2007. Identification of antioxidants in essential oil of radix *Angelicae s*inensis using HPLC coupled with DAD-MS and ABTS-based assay. J. Agric. Food Chem. 55:3358-3362.

Malencic, D., M. Popovic and J. Miladinovic. 2007. Phenolic content and antioxidant properties of soybean [*Glycine max* (L.) Merr.] seeds. Molecules 12:576-581.

Malencic, D., Z. Maksimovic, M. Popovic and J. Miladinovic. 2008. Polyphenol contents and antioxidant activity of soybean seed extracts. Bioresour Technol. 99:6688-6691.

Medina, I., J.M. Gallardo, M.J. Gonzalez, S. Lois and N. Hedges. 2007. Effect of molecular structure of phenolic families as hydroxycinnamic acids and catechins on their antioxidant effectiveness in minced fish muscle. J. Agric. Food Chem. 55:3889-3895.

Mezei, O., W.J. Banz, R.W. Steger, M.R. Peluso, T.A. Winters and N. Shay. 2003. Soy isoflavones exert antidiabetic and hypolipidemic effects through the PPAR pathways in obese Zucker rats and murine RAW 264.7 cells. J. Nutr. 133:1238-1243.

Naim, M., B. Gestetner, A. Bondi and Y. Birk. 1976. Antioxidative and antihemolytic activities of soybean isoflavones. J. Agric. Food Chem. 24:1174-1177.

Nanjo, F., K. Goto, R. Seto, M. Suzuki, M. Sakai and Y. Hara. 1996. Scavenging effects of tea catechins and their derivatives

on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radic. Biol. Med. 21:895-902.

Ozcelik, B., J.H. Lee and D.B. Min. 2003. Effects of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. J. Food Sci. 68:487-490.

Qian, Z.M., J. Guan, F.Q. Yang and S.P. Li. 2008. Identification and quantification of free radical scavengers in Pu-erh tea by HPLC-DAD-MS coupled online with 2,2'-azinobis(3-ethyl benzthiazolinesulfonic acid) diammonium salt assay. J. Agric. Food Chem. 56: 11187-11191.

Riedl, K.M., J.H. Lee, M. Renita, S.K. St Martin, S.J. Schwartz and Y. Vodovotz. 2007. Isoflavone profiles, phenol content, and antioxidant activity of soybean seeds as influenced by cultivar and growing location in Ohio. J. Sci. Food Agric. 87:1197-1206.

Sakthivelu, G., M.K.A. Devi, P. Giridhar, T. Rajasekaran, G.A. Ravishankar, M.T. Nikolova, G.B. Angelov, R.M. Todorova and G.P. Kosturkova. 2008. Isoflavone composition, phenol content, and antioxidant activity of soybean seeds from India and Bulgaria. J. Agric. Food Chem. 56:2090-2095.

So, E.H., J.H. Kuh, K.Y. Park and Y.H. Lee. 2001. Varietal difference of isoflavone content and antioxidant activity in soybean. Kor. J. Breed Sci. 33:35-39 (in Korean).

Stangl, V., M. Lorenz and K. Stangl. 2006. The role of tea and tea flavonoids in cardiovascular health. Mol. Nutr. Food Res. 50:218-228.

Stewart, A.J., W. Mullen and A. Crozier. 2005. On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea. Mol. Nutr. Food Res. 49:52-60.

Tsukamoto, C., S. Shimada, K. Igita, S. Kudou, M. Kokubun, K. Okubo and K. Kitamura. 1995. Factors affecting isoflavone content in soybean seed: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J. Agric. Food Chem. 43:1184-1192.

Vasco, C., K. Riihinen, J. Ruales and A. Kamal-Eldin. 2009. Phenolic compounds in *Rosaceae* fruits from Ecuador. J. Agric. Food Chem. 57:1204-1212.

Wang, K.J., C.R. Yang and Y.J. Zhang. 2007. Phenolic antioxidants from Chinese toon (fresh young leaves and shoots of *Toona sinensis*). Food Chem. 101:365-371.

Ryu, Y.H., Y.H. Lee, K.Y. Park, H.T. Yun, U.H. Kim, J.E. Lee and C.K. Lee. 2001. Standard Farming Handbook 116: Soybean cultivation. Rural Development Administration.

Zeeb, D.J., B.C. Nelson, K. Albert and J.J. Dalluge. 2000. Separation and identification of twelve catechins in tea using liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry. Anal. Chem. 72:5020-5026.

Zielinski, H. and H. Kozlowska. 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. J. Agric. Food Chem. 48:2008-2016.

(Received 11 September 2012 ; Revised 5 June 2013 ; Accepted 16 October 2014)