

Rapid High Performance Liquid Chromatographic Quantification of Major Isoflavones in Soybeans and Soybean Pastes

Won-Chan Kim, Soon-Ho Kwon, In-Koo Rhee, Jong-Moon Hur, Hyun-Hee Jeong, Sun-Ha Choi¹, Kyung-Bok Lee¹, Young-Hwa Kang and Kyung-Sik Song*

Division of Applied Biology & Chemistry, College of Agriculture & Life Sciences, Kyungpook National University, Daegu 702-701, Korea

¹Department of Biochemistry, School of Medicine, Konyang University, Nonsan, Choongnam 320-711, Korea

Abstract A simple HPLC quantification method was developed for genistein, genistin, daidzein, and daidzin in soybeans and soybean products. The procedure used a 4.6×100 mm Chromolith® RP-18e column with a mobile phase of 1% HOAc in 20% MeOH to 1% HOAc in 80% MeOH for 10 min. The injection volume was 2 µL at a flow rate of 2 mL/min. Detection was carried out under UV at 254 nm. Under these conditions, the major isoflavones daidzein, daidzin, genistein, and genistin in soybean and soybean pastes were eluted within 7 min with baseline separation. Optimal extraction of the above four major isoflavones was achieved when 40 g of soybean or soybean paste was refluxed in 100 mL of 95% ethanol for 2 hr. Ten different soybean cultivars and nine commercial soybean pastes were analyzed by this method. The total isoflavone content was highest in the cultivar Somyung (2,497 µg/g dry weight). The isoflavone content in soybean pastes varied widely from manufacturer to manufacturer (an almost five-fold difference between the highest and lowest values). Such variations were presumably due to differences in fermentation conditions, type of soybeans used, and levels of such additives as starch and salt.

Keywords: high performance liquid chromatography, soybean, isoflavone, isoflavone glycoside, quantification

Introduction

Soybeans have been known to be a rich source of isoflavones like daidzein and genistein, and their glycosides daidzin and genistin. The soybean isoflavones have received a great deal of attention for their anti-carcinogenic, antioxidant, antimutagenic, and estrogenic effects (1-5). In addition, epidemiological studies have indicated that the consumption of soybeans may be associated with a reduced risk of cancer (6, 7), cardiovascular disease (8), and osteoporosis (9). In this sense, the quality of soybeans and soybean products tends to be evaluated by their isoflavone content. The quantification of major soybean isoflavones has been carried out by gas-chromatography (GC), GC-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and capillary electrophoresis (CE) (10-14). However, known methods require about 40-100 min of analysis time and complicated pretreatment procedures such as hydrolysis, extraction, filtration, and evaporation. Recently, a fast and simple method using automated on-line in-tube solid-phase microextraction (SPME) was developed (15). Although it seemed to be a very efficient method, it required specialized devices such as SPME-HPLC. Therefore, it is necessary to develop more efficient and simpler analytical methods. This report describes a simple HPLC quantification method for the major soybean isoflavones, the optimal pretreatment conditions for HPLC analysis, and the isoflavone content

of soybeans and soybean pastes distributed in Korean markets.

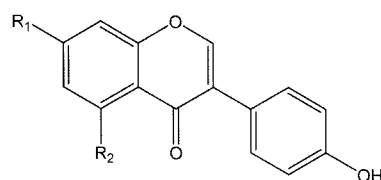
Materials and Methods

Chemicals, reagents, and soybean samples Daidzin (1), genistin (2), daidzein (3), and genistein (4) were purchased from Sigma (St. Louis, MO, USA). The purity of each sample was checked by HPLC-PDA analysis. Methanol and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). Spectrophotometry-grade glacial acetic acid was obtained from Wako Co. (Osaka, Japan). All other chemicals were of analytical reagent grade. Different varieties of soybeans were cultivated and harvested in 2001 from Yeongnam Agricultural Research Institute, NICS, Kyungnam, Korea. Commercial soybean pastes were purchased from commercial markets in Daegu, Korea.

Pretreatment of soybean samples In order to establish optimal pre-treatment conditions for HPLC analysis, dried Somyung soybeans (10-80 g) were finely powdered with mortar and pestle. Bean powder was refluxed with 100 mL ethanol (EtOH) in a water bath for appropriate periods of time (30-240 min). The extracted solution was filtered through filter paper and the volume of filtrate was adjusted to 100 mL with EtOH. One mL of each sample was taken to be filtered through a membrane filter (0.45 µm) and an aliquot of filtrate (2 µL) was injected for HPLC analysis. For quantifying isoflavones in soybeans and soybean products, 40 g sample/100 mL 95% EtOH was extracted for 2 hr. Prior to extraction, soybean pastes were spread onto aluminum foil and dried in an oven (110°C) until a constant weight (within ±1%) was obtained. The HPLC

*Corresponding author: Tel: 82-53-950-5715; Fax: 82-53-956-5715
E-mail: kssong@knu.ac.kr

Received July 12, 2005; accepted November 17, 2005



- 1, Daidzin: $R_1=OGlc$, $R_2=H$
- 2, Genistin: $R_1=OGlc$, $R_2=OH$
- 3, Daidzein: $R_1=OH$, $R_2=H$
- 4, Genistein: $R_1=OH$, $R_2=OH$

Fig. 1. The structures of isoflavones.

analysis procedures for soybean pastes were exactly the same as those for Somyung soybeans.

HPLC analysis HPLC was performed on a Jasco HPLC (Easton, MD, USA) consisting of a PU-2080 binary pump, an AS-2055 autosampler, and an MD-2010 photodiode array (PDA) detector. The columns tested were a 4.6×100 mm Merck Chromolith RP-18e (Merck), a 3.9×150 mm Waters Novapak C_{18} (Palo Alto, CA, USA), a 3.9×150 mm Waters μ Bondapak C_{18} , a 4.6×150 mm, Agilent Eclipse \times DB- C_{18} (USA), and a 4.6×150 mm Agilent Hypersil ODS. The mobile phase consisted of 1% acetic acid in water with 1% acetic acid in methanol. Elution was carried out with a gradient system of 20 to 80% methanol solution for ten minutes at room temperature. The flow rate was 2 mL/min and detection was carried out under UV at 254 nm. From ten mM stock solutions of **1-4**, serial dilutions of standard solutions were prepared. All standard samples were dissolved in 95% ethanol (EtOH) and stored at or below 4°C until used. Calibration curves were obtained by analyzing four different concentrations of standards in duplicate. The calibration curves of standard isoflavones **1-4** were linear ($r=0.997$ for **1**, 0.998 for **2**, 0.997 for **3**, and 1.000 for **4**) in the range of 0.1-1.5 ng under the above HPLC conditions.

Results and Discussion

Recent papers have dealt with the separation of phytoestrogens in soybeans, soy foods, and human body fluids (16, 17). In some of these works, the proposed analytical methods involved hydrolyzation of samples and quantification of the aglycones obtained. These methods, however, can provide only limited information on the quality of soybeans because some isoflavone glycosides also have important biological activities (18). Therefore, we used a non-hydrolytic method, and it was expected that HPLC would allow separation both of the major isoflavone aglycones and their glycosides. Even previous quantitative studies revealed that fresh soybeans contained large amounts of isoflavones that were mainly available as malonate conjugates (e.g. glycoside malonates of daidzein and genistein, glycoside malonates in fresh plants have been known to be unstable during drying and processing),

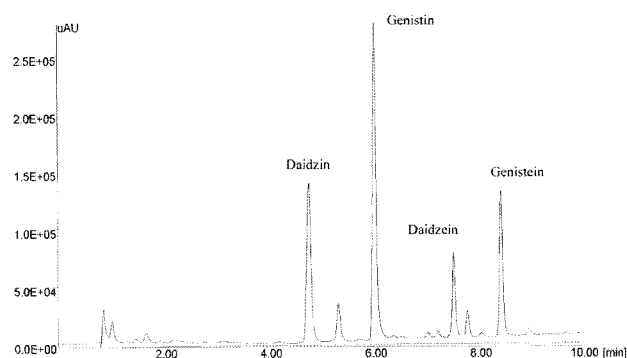


Fig. 2. Typical HPLC profile of soybean (Somyung) extract. The column used was a 4.6×100 mm Chromolith RP-18e®.

making it very difficult to procure and maintain pure standards (19-22). In addition, malonate conjugates are less biologically active (23, 24). Clinical and preclinical studies found that the major soybean isoflavones like daidzein, genistein, and their corresponding glucosides may be absorbed or further metabolized to many specific bio-available metabolites including equol (1, 23-24). Therefore, HPLC analysis and pretreatment conditions were optimized for the four major isoflavones: genistein, genistin, daidzein, and daidzin.

In order to find an optimal stationary phase for isoflavone quantification, several commercially available HPLC columns were tested (data not shown). Among them, satisfactory chromatographic separation was achieved on Chromolith RP-18e using a methanol-water-acetic acid solvent system. The base line separation of four major isoflavones was made within seven minutes under the HPLC conditions described in the Materials and Methods section. Figure 2 shows a chromatogram of typical soybean extract from cultivar Somyung under UV detection at a wavelength of 254 nm. The retention time (t_R)'s of compound **1-4** were 7.56, 4.80, 8.46, and 6.05 min, respectively. The peaks were identified by co-chromatographic analysis and by comparing their t_R 's and corresponding UV spectra in PDA with those of standards. The effects of extraction time and solvent-solute ratio were investigated in order to optimize the extraction of daidzein, genistein, daidzin, and genistin. Ninety five % ethanol was selected as an extraction solvent instead of acetonitrile because of its lower boiling point, lower toxicity, and lower degree of environmental impact as well as economical and practical reasons (other organic solvents such as chloroform, methanol, ether, and acetone are seldom used in food processing) (25). The yield increased dramatically after 2 hr of extraction, and remaining constant for the next 2 hr (Fig. 3). In addition, the optimum solute-solvent ratio for the extraction of soybean flavonoids was 40 g in 100 mL EtOH [1:2.5 (g/mL)] (Fig. 4).

The methods developed in this study were applied to quantifying isoflavone content in soybeans and soybean pastes commercially distributed in Korea. As shown in Table 1, depending on the cultivar, the total amount of major isoflavones varied from 524 to 2,497 μ g/g dry weight. These results were in agreement with previous findings (345-5,394 μ g/g fresh weight of seed flour) (26-

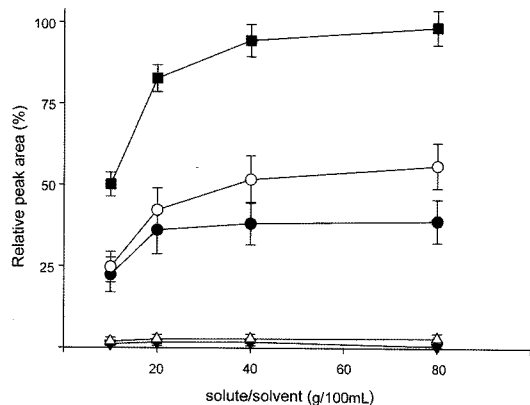


Fig. 3. Extraction efficiency of isoflavones according to the solute-solvent ratio. Various concentrations of soybean (Somyung) in ethanol were extracted in a water bath for 3 hr, and then the extracts were analyzed in duplicate under the conditions described in Fig. 2. ● = daidzin, ○ = genistin, ▼ = daidzein, ▽ = genistein, ■ = total.

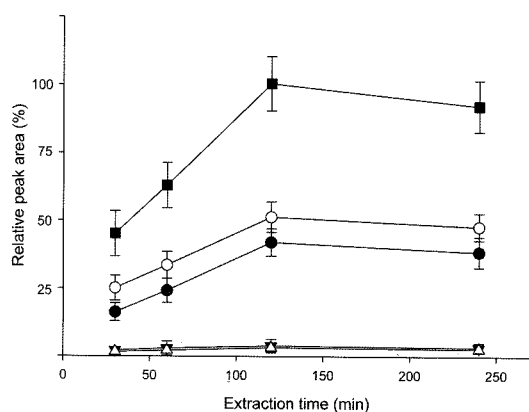


Fig. 4. Extraction efficiency of isoflavones according to the extraction time. Forty grams of soybeans (Somyung) were extracted in 100 mL of 95% ethanol for 30-240 min, and each extract was analyzed in duplicate under the condition described in Fig. 2. ● daidzin, ○ = genistin, ▼ = daidzein, ▽ = genistein, ■ = total.

28). Wang *et al.* (27) suggested that year and location of cultivation, as well as soybean variety, could have an effect on the isoflavone content of soybeans. They insisted that year of harvest seemed to have a much greater influence on isoflavone content (27). However, all soybeans used in this experiment had been harvested in the same year, thus variations in isoflavone content can be assumed to originate mainly from cultivar differences. Among the samples tested, the Somyung soybean cultivar showed the highest levels of aglycone, glycoside, and total isoflavone (Table 1). These have been known to be the most biologically active isoflavones found in soybean seeds (29). The levels of major isoflavones in soybean pastes showed a lot of variation between manufacturers, with an almost five-fold difference between the highest and the lowest values (Table 2). This is presumably due to differences in fermentation conditions, soybean cultivars used, and amounts of additives like starch or salt. Due to the unclear package label information, levels of soybean material and other accessory ingredients could not be

Table 1. Isoflavone contents in different soybean cultivars

Cultivar	Glycoside*		Aglycone*		Total*
	Daidzin	Genistin	Daidzein	Genistein	
Jinyul	217±11	256±2	trace	52±1	524
Manri	352±3	468±2	373±2	217±2	1,410
Daewon	374±10	57±3	448±3	239±1	1,568
Dawon	105±22	230±2	144±1	162±1	641
Jinpoom	328±1	395±1	391±1	184±3	1,298
Jangwon	213±4	334±9	301±5	210±6	1,057
Jangsoo	419±21	483±10	249±6	227±3	1,378
Danbaek	244±5	333±11	259±3	222±5	1,057
Somyung	758±10	919±8	509±7	311±2	2,497
Gumeunkong 1	179±9	282±12	259±1	212±1	932

*mg/g.

Table 2. Isoflavone contents in soybean pastes distributed in Korean markets

Manufacturer	Glycoside*		Aglycone*		Total*
	Daidzin	Genistin	Daidzein	Genistein	
A	108±2	150±2	505±3	685±5	1,493
B	249±1	227±3	590±9	731±2	1,797
C	312±2	377±2	317±1	481±3	1,486
D	363±2	405±3	604±2	766±1	2,137
E	140±3	270±5	809±2	1,028±2	2,248
F	162±3	362±6	1,068±1	1,000±2	2,591
G	99±1	110±4	1,085±3	1,034±3	2,328
H	trace	trace	924±5	386±2	1,310
I	trace	trace	301±2	240±4	541

*mg/g.

determined in the commercial soybean pastes tested. As a result, we could not calculate the exact amount of soybean-derived isoflavones in the pastes.

Many approaches have been taken to quantifying isoflavone content in soybeans and other plants. These employed various solvent systems and columns. However, factors like long analysis time (more than 40 min) (14), complicated pretreatment (30), and need for special equipment (15) have rendered their results difficult to apply to a practical industrial field. The method described here is simple, rapid, and even economical for the quantification of major isoflavone content in soybeans and soybean products. It is expected that this method will provide a useful solution to controlling the quality of soybeans and soybean products.

Acknowledgments

This work was supported by a Grant from the Ministry of Agriculture and Forestry, Korea.

References

1. Tham DM, Gardner CD, Haskell WL. Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.* 83: 2223-2235 (1998)
2. Nam HY, Min SG, Shin HC, Kim HY, Fukushima M, Han KH, Park WJ, Choi, KD, Lee CH. The protective effect of isoflavone extracted

- from soybean paste in free radical inhibitor treated rats. *Food Sci. Biotechnol.* 14: 586-592 (2005)
3. Bingham SA, Atkinson C, Liggins J, Bluck L, Coward A. Phytoestrogens: where are we now? *Br. J. Nutr.* 79: 393-406 (1998)
 4. Bingham SA. Diet and colorectal cancer prevention. *Biochem. Soc. Transact.* 28: 12-16 (2000)
 5. Jacobsen BK, Knutsen SF, Fraser GE. Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). *Cancer Causes Control.* 9: 553-557 (1998)
 6. Bradlow HL, Sepkovic DW. Diet and breast cancer. *An. New York Acad. Sci.* 963: 247-267 (2002)
 7. Alkel DL, Sirtori CR, Muanza DN, Vasquez A, Cannell J, Grant WP, van der Schouw YT, Grobbee DE, Kok L, Kreijkamp-Kaspers S. Isoflavones and postmenopausal women. *J. Am. Med. Assoc.* 292: 2336-2337 (2004)
 8. van der Schouw YT, de Kleijn MJ, Peeters PH, Grobbee DE. Phytoestrogens and cardiovascular disease risk. *Nutr. Metab. Cardiovasc. Dis.* 10: 154-67 (2000)
 9. Anderson JJ, Garner SC. Phytoestrogens and bone. *Bailliere's Clin. Endocrinol. Metab.* 12: 543-557 (1998)
 10. Ghosh P, Fenner GP. Improved method for gas chromatographic analysis of genistein and daidzein from soybean (*Glycine max*) seeds. *J. Agric. Food Chem.* 47: 3455-3456 (1999)
 11. Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA. Daidzein and genistein contents of vegetables. *Br. J. Nutr.* 84: 717-725 (2000)
 12. Nurmi T, Mazur W, Heinonen S, Kokkonen J, Adlercreutz H. Isoflavone content of the soy based supplements. *J. Pharm. Biomed. Anal.* 28: 1-11 (2002)
 13. Satterfield M, Black DM, Brodbelt JS. Detection of the isoflavone aglycones genistein and daidzein in urine using solid-phase microextraction-high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B.* 759: 33-41 (2001)
 14. Bala S, Uniyal GC. High-performance liquid chromatographic analysis of genistein and other isoflavonoids from heartwood of *Ougeinia dalbergioides*. *Phytochem. Anal.* 13: 211-214 (2002)
 15. Mitani K, Narimatsu S, Kataoka H. Determination of daidzein and genistein in soybean foods by automated on-line in-tube solid-phase microextraction coupled to high-performance liquid chromatography. *J. Chromatogr. A.* 986: 169-177 (2003)
 16. Williamson AP, Wähälä K, Williamson G. Identification and quantification of polyphenol phytoestrogens in foods and human biological fluids. *J. Chromatogr. B.* 777: 93-109 (2002)
 17. Kao TH, Chen BH. An improved method for determination of isoflavones in soybean powder by liquid chromatography. *Chromatographia* 56: 423-430 (2002)
 18. Coward L, Smith M, Kirk M, Barnes S. Chemical modification of isoflavones in soy foods during cooking and processing. *Am. J. Clin. Nutr.* 68: 1486S-1491S (1998)
 19. Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC mass spectrometry. *J. Agric. Food Chem.* 42: 2466-2474 (1994)
 20. Simmonne AH, Smith M, Weaver DB, Vail T, Barnes S, Wei CI. Retention and changes of soy isoflavones and carotenoids in immature soybean seeds (Edamame) during processing. *J. Agric. Food Chem.* 48: 6061-6069 (2000)
 21. Coward L, Smith M, Kirk M, Barnes S. Chemical modification of isoflavones in soy foods during cooking and processing. *Am. J. Clin. Nutr.* 68: 1486S-1491S (1998)
 22. Mahungu SM, Diaz-Mercado S, Li J, Schwenk M, Singletary K, Faller J. Stability of isoflavones during extrusion processing of corn/soy mixture. *J. Agric. Food Chem.* 47: 279-284 (1999)
 23. Setchell KDR, Cassidy A. Dietary isoflavones: biological effects and relevance for human health. *J. Nutr.* 129: 758S-767S (1999)
 24. Fang, NB, Yu SG, Badger TM. Characterization of isoflavones and their conjugates in female rat urine using LC/MS/MS. *J. Agric. Food Chem.* 50: 2700-2707 (2002)
 25. Murphy PA, Baruna K, Hauk CC. Solvent extraction selection in the determination of isoflavones in soy foods. *J. Chromatogr. B.* 777: 129-138 (2002)
 26. Wang HJ, Murphy PA. Isoflavone contents in commercial soybean foods. *J. Agric. Food Chem.* 42: 1666-1673 (1994)
 27. Wang HJ, Murphy PA. Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* 42: 1674-1677 (1994)
 28. Wu Q, Wang M, Sciarappa WJ, Simon JE. LC/UV/ESI-MS analysis of isoflavones in Edamame and tofu soybeans. *J. Agric. Food Chem.* 52: 2763-2769 (2004)
 29. Dixon RA, Ferreira D. Genistein. *Phytochemistry* 60: 205-211 (2002)
 30. Krenn L, Unterrieder I, Ruprechter R. Quantification of isoflavones in red clover by high-performance liquid chromatography. *J. Chromatogr. B.* 777: 123-128 (2002)