

Enhanced extraction of isoflavones from Korean soybean by ultrasonic wave

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Abstract—The amounts of isoflavones extracted from Korean soybean by various ultrasonic waves were compared using 60% aqueous ethanol solution. The effect on extraction yield of variations in solvent composition, temperature, and extraction time was investigated. The experimental results confirmed that ultrasonic waves are a desirable method to extract isoflavones from Korean soybean. The highest yield of aglycone isoflavones was obtained by ultrasonic waves with a frequency of 20 KHz and an extraction time of 10 min, which produced yields of glycoside and aglycone isoflavones three-fold greater than those by dipping method.

Key words: Korean Soybean, Glycoside, Aglycone, Ultrasonics Wave, HPLC

INTRODUCTION

Isoflavones are naturally occurring plant chemicals belonging to the phytoestrogen and are reported to have therapeutic or preventive effects on a range of hormone-dependent conditions, including cancer, cardiovascular disease, osteoporosis, and menopausal symptoms [Kim et al., 1999; Lee et al., 2005]. Isoflavones are a flavonoid subgroup found in several plants. In soybeans, they are present in 12 main forms including genistin, glycitin, daidzin and their respective acetyl, malonyl and aglycon derivation [Griffith et al., 2001; Lee et al., 2003; Kim et al., 2005]. Isoflavones in soybeans occur both free and bound to glucose moieties (glycones). Aglycone isoflavones have higher antioxidant activity than bound phenolics, and are absorbed faster and in higher amounts [McCue et al., 2004]. Recently, other good effects of soybeans have been studied. They may contribute to many of the health benefits attributed to soybean foods [Wu et al., 2001; Dixon et al., 2002; Choi et al., 2004]. Typically, isoflavones have been extracted by using aqueous methanol (MeOH), ethanol (EtOH), or acetonitrile (MeCN) solutions. A modern method used to release the bioactive constituents from herbs is ultrasonic enhanced solvent extraction [Rostagno et al., 2003]. The mechanical effects of ultrasonic have been recognized and studied for many years. There have been numerous reports on the application of high intensity or power ultrasonic in the extraction of various phytochemicals, such as alkaloids, flavonoids, polysaccharides, proteins, and essential oils from various parts of plant and plants seed [Melecchi et al., 2005]. Ultrasonics have also been applied for the intensity and frequency of extraction processing [Tuziuti et al., 2004; Iida et al., 2005]. The propagation and interaction of sound waves alters the physical and chemical properties of the materials [Haizhou et al., 2004]. Ultrasonic science and technology are becoming a growing research field because of a wide range of emerging (functional food) applications in chemical synthesis, therapeutics, environmental protection, electrochemistry, processing of food, and processing

of solids and liquid [Lee et al., 2005; Gachagan et al., 2004; Iida et al., 2005; Moussatov et al., 2005]. In the case of raw plant issues, ultrasonics have been suggested to disrupt plants cell walls, thereby facilitating the release of extractable compounds and enhancing mass transport of the solvent from the continuous phase into plant cells [Yasui et al., 2005]. Ultrasonics also exert a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phase. Ultrasonics have great potential for uses in a wide variety of processes in the chemical and applied industries [Haizhou et al., 2004; Sez et al., 2005].

In this work we investigated the different extraction methods that applied dipping and ultrasonics. The feasibility of using Korean soybean and fermented products as a source for the isoflavones was studied. The controlled experimental variables were temperature (25, 40, 60, and 80 °C), frequency (20, 40, 68, and 110 KHz), and extraction time (10 min). The experiments were performed using high performance liquid chromatography (HPLC).

EXPERIMENT

1. Reagents

The standard chemicals of daidzin, genistin, daidzein and genistein were obtained from Sigma. Co. (USA), while acetyl daidzin, malonyl genistin, glycitin and glycitein were from LC Laboratories (Woburn, MA, USA). HPLC-grade ethanol, methanol and acetonitrile were purchased from J. T. Baker (USA). The Korean soybean was from Mejoo & Cellist Co. (Kangwon Do, Korea). The twice distilled water was filtered by a pump (Division of Millipore, Waters, Milford, MA, USA) and filter (FH- 0.2 µm, Waters, Milford, MA, USA).

2. Sample Preparation

Samples were prepared by dissolving 2 mg of the standard chemicals (daidzin, glycitin, genistin, acetyl daidzin, malonyl genistin, daidzein, glycitein and geinstein) in 4 ml of ethanol and adjusting the concentration of the isoflavones to 500 ppm.

3. Solvent Extraction

5 g samples of the powder from the Korean soybean containing

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the isoflavones were loaded in 100 ml of an aqueous ethanol solution (40, 60%), pure water and ethanol 100 ml. The effects of variations in time and temperature and in the temperature of the dipping method (25, 40, 60, and 80 °C) were measured. The following experimental variables were applied: ultrasonic frequency of 20, 40, 68, and, 110 KHz, intensity of 100 W, temperature of 25 °C and ultrasonic frequency of 20 KHz for a time of 10 and 30 sec, and 1, 3, 5, 10, 15, and 20 min. Each extract underwent decompression filtration and decompression concentration to evaporate the solvent. The extraction was applied by ultracentrifuge for 90 min at 15,000 RPM. The solution was filtered through a 0.2 µm membrane filter prior to HPLC analysis.

4. Ultrasonic System

Two types of ultrasonic system were used: a horn-type ultrasonic (frequency 20 KHz, output power 400 Watt MAX, dimension 341 L×110 W×110 H, main source AC 220 V, Mirae Ultrasonic Tech. Co. Korea) and a standing-wave type system (frequency 40, 68, 110 KHz, output power 300 Watt MAX, dimension 35 L×12 W×12 H, main source AC 220 V, 5 L, Mirae Ultrasonic Tech. Co. Korea). An ultracentrifuge separator (Hanil Science Industrial, Model : Micro 17 R Pluses, Max speed) : 17,000 RPM, Korea) was also used.

5. HPLC System

The experiments were performed with a Waters HPLC system equipped with a 600E pump 486 detector, injector 20 µl sample loop (Rheodyne, USA) and Chromate 3.0 data acquisition system (Interface Eng.). Sufficient times were allowed for the stabilization of the column and detector signal after each injection, and the solvents in the reservoirs were continuously stripped with helium to degas the mobile phase. The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. The Korean soybean was sieved at 32 µm, ground in a dry state for 2 min in a food mixer (Hannil Mixer FM-909 T, 220 W, 1.3 A) and then underwent ultracentrifugation (Hannil Science Industrial, Model : Micro 17 R Plus, Korea). The isoflavones, daidzin, glycitin, genistin, acetyl daidzin, malonyl genistin, daidzein, glycitein and genistein, were identified by LC-MS (UATTRO LC Triple Quadrupole Tandem Mass Spectrometer). The chromatographic columns used in this experiment are commercially available; one was obtained from RS-tech (0.46×25 cm, 5 µm, C₁₈, Daejeon, Korea), and the other was a YMC column (0.46×25 cm, 5 µm, C₁₈, YMC-pack Pro). The injection volume was 20 µl, and the flow rate of the mobile phase was 1.0 ml/min. The wavelength of the UV detector was fixed at 254 nm. The composition of the mobile phases was reservoir A (water/acetic acid=99.9/0.1, vol%) and reservoir B (ace-

tonitrile/acetic acid=99.9/0.1, vol%). The run time was 50 min and the solvent program was the linear gradient method (85 : 15-65 : 35, A : B vol%).

RESULTS AND DISCUSSIONS

In this study, the effect of variations in solvent composition (%), temperature (°C), frequency (KHz) on the yield of isoflavones extracted from Korean soybean was investigated. We investigated the yields of the glycosides, daidzin, glycitin, genistin, acetyl daidzin, and malonyl genistin, and of the aglycones, daidzein, glycitein and genistein, of the isoflavones from the soybeans by different extraction methods. In order to calculate the amount of isoflavones extracted from the soybeans, a calibration curve needs to be drawn. The area (under the curve) method was used in this experiment. First, the solution concentration was fixed at 0.5 mg/ml and varying sample volumes (5 µl to 20 µl) were injected into the HPLC system. The peak areas for the sample of each concentration were obtained, and linear regression was applied to the calibration curve. Different methods were used for the determination of the isoflavones. Although HPLC is the most common procedure, different research groups have used different methodologies for the extraction of isoflavones, using solvents such as methanol and ethanol, and various temperatures and lengths of time for the extraction. The amount of isoflavones extracted in the chromatogram was calculated by regression analysis. The parameters of the calibration curves of the isoflavones were daidzin $Y=4.22 \times 10^{-10} X$, glycitin $Y=2.92 \times 10^{-10} X$, genistin $Y=2.34 \times 10^{-10} X$, acetyl daidzin $Y=3.61 \times 10^{-10} X$, malonyl genistin $Y=3.81 \times 10^{-10} X$, daidzein $Y=2.58 \times 10^{-10} X$, glycitein $Y=3.72 \times 10^{-10}$ and genistein $Y=2.11 \times 10^{-10} X$. The correlation coefficient (r^2) was higher than 0.98 in each case.

$$r^2 = 1 - \frac{\sum_{i=1}^n (y_i - \langle y \rangle)^2}{\sum_{i=1}^n (y_i - y(x_i))^2}, \quad \langle y \rangle = \frac{\sum y_i}{N} \quad (1)$$

Where X means the peak area (mV×sec) under the curve in the chromatogram and Y means the amount (µg) of the isoflavones according to the injection volume for each standard sample. The various experimental variables were analyzed by a commercially available C₁₈ column. The mobile phases were composed of water with acetic acid and acetonitrile on gradient mode. The mobile phase composition of reservoir B was linearly increased from 15 to 35 vol%. In the RS-tech column (0.46×25 cm, 5 µm, C₁₈), a 20 µl sample of Korean soybean was injected. The isoflavones of glycosides, daid-

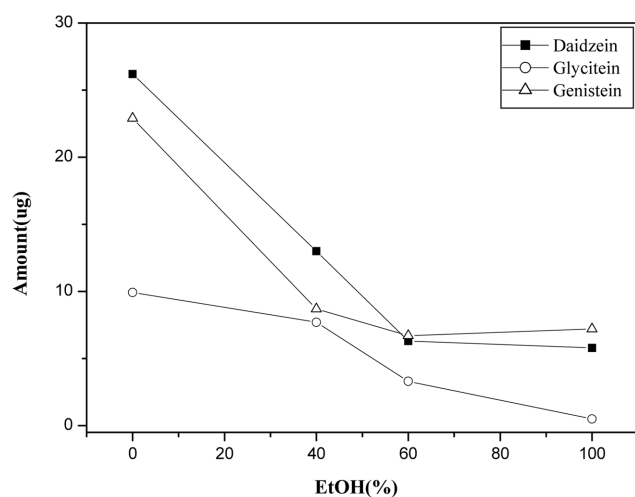
Table 1. Content of some isoflavones from Korean soybean by ultrasonic wave and dipping (condition: room temperature)

Substance		Daidzin	Glycitin	Genistin	Acetyl daidzin	Malonyl genistin	Daidzein	Glycitein	Genistein
Extraction condition									
Dipping		125.0	14.7	260.7	13.5	493.0	6.3	3.2	6.7
Ultrasonic	20 (KHz)	388.1	72.3	700.50	41.42	1098.6	23.9	15.0	15.1
	40 (KHz)	287.6	67.4	813.9	29.7	1118.9	12.5	13.0	8.7
	68 (KHz)	196.4	31.0	841.1	28.3	1200.2	9.3	6.3	6.8
	110 (KHz)	88.11	28.6	494.5	13.6	941.9	6.5	4.7	5.6

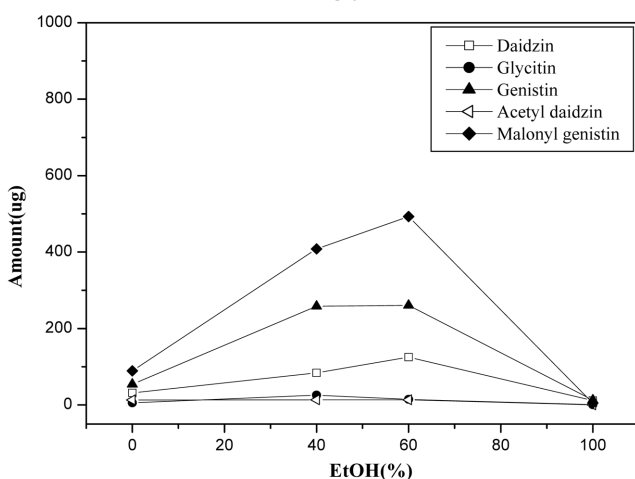
(unit: µg, loading amount=1 g of sample)

zin, glycitin, genistin, acetyl daidzin, and malonyl genistin, and of the aglycones, daidzein, glycitein and geinstein, were identified by standard components. The various experimental variables were extraction solvent (aqueous ethanol solution 40, 60%, pure water and ethanol), dipping temperature (25, 40, 60, and 80 °C), frequency (20, 40, 68, and 110 KHz) and extraction time (10 and 30 sec, and 1, 3, 5, 10, 15, and 20 min). Table 1 shows the content of the isoflavones extracted from Korean soybean by ultrasonic wave and dipping method.

Fig. 1 shows the effect of extraction solvent volume on the extraction of isoflavones. At a room temperature dipping time of 10 min, the extraction percentage of isoflavones increased with increasing solvent volume. The major types of isoflavones were genistin and malonyl genistin. The chemical structure of isoflavones contains both hydrophobic and hydrophilic regions. Isoflavones are soluble in aqueous organic solvent. They are best solubilized in 60% aqueous ethanol. After experimental processing, 60% aqueous ethanol was employed. Fig. 2 shows the effect of temperature (25, 40, 60,

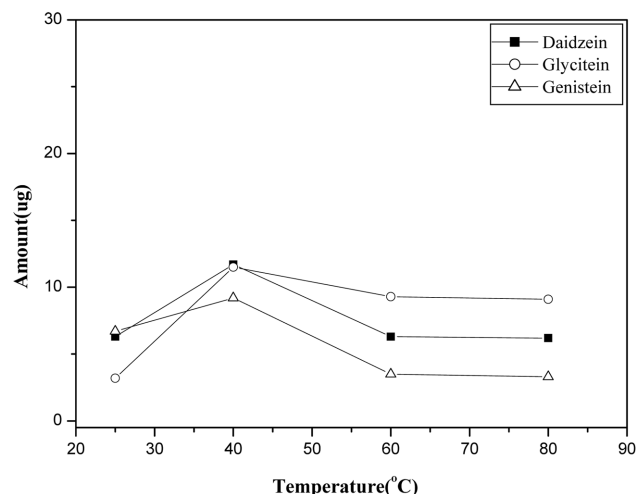


(a) Aglycones

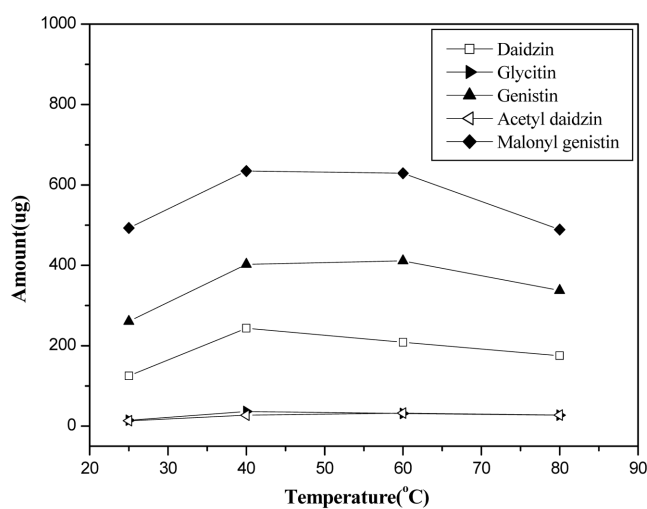


(b) Glycosides

Fig. 1. Extracted amount of some isoflavones with different extraction solvent composition (Aqueous ethanol solvent 40, 60, pure water, ethanol 100% with extraction dipping time: 10 min).



(a) Aglycones



(b) Glycosides

Fig. 2. Extracted amount of some isoflavones with different temperature composition (Temperature 25, 40, 60, 80 °C with extraction dipping time: 10 min).

and 80 °C) on the extraction of isoflavones from Korean soybean. Extraction dipping time was set at 10 min. The amounts of the total glycoside and aglycone isoflavones were 906.9 and 16.2 µg at 25 °C, 1,344.6 and 32.4 µg at 40 °C, 1,312.5 and 19.0 µg at 60 °C, and 1,056.7 and 18.6 µg at 80 °C, respectively. Extraction efficiency was excellent at 40 °C. Also, the effect of different frequencies of ultrasonic waves on the extracted amounts of isoflavones from Korean soybean was studied. The effects of the collapse of the cavitation bubble on the cell walls of soybean during ultrasonic extraction can be described by the following reaction. In order to achieve high extraction efficiency, optimization of ultrasonic operational parameters (solvent polarity, sample particle size) according to a specific plant matrix (leaves, stems, and seed) is very important [Meleccchi et al., 2005]. In this case, isoflavone yield was reduced with increasing frequency. However, aglycones showed excellent extraction performance at 20 KHz. On collapse, the transient cavitation bubble produces a very high temperature (80 °C) pressure pulse and the cavity explodes with strong energy release.

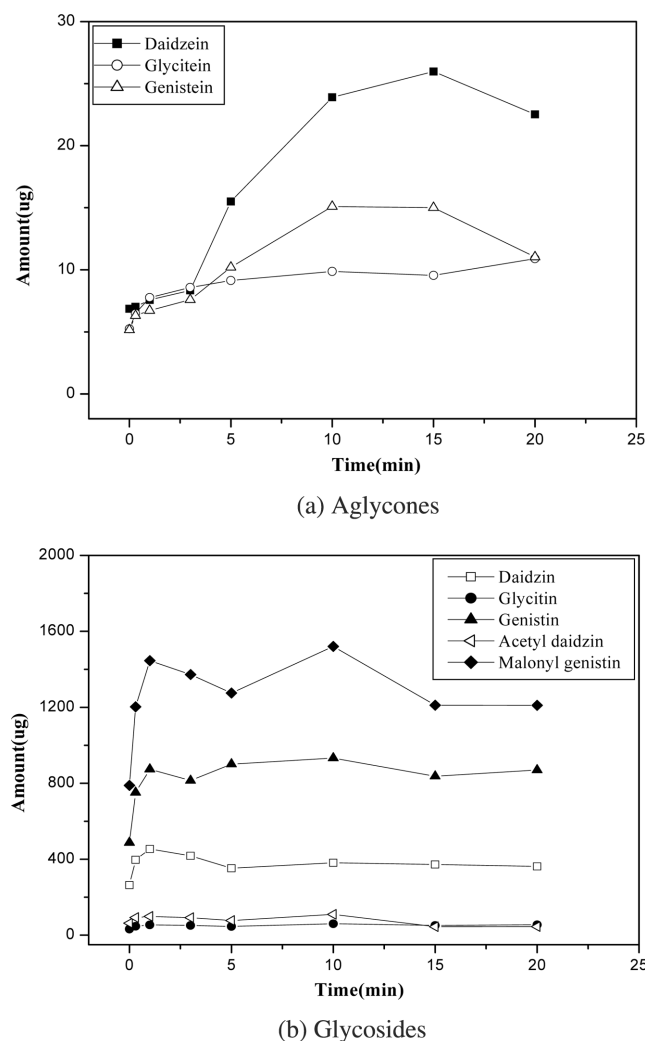


Fig. 3. Efficiency extraction of some isoflavones (Frequency: 20 KHz, with extraction time: 10, 30 sec, 1, 3, 5, 10, 15, 20 min).

Fig. 3 shows the effect of extraction time on the extracted amount of isoflavones with 20 KHz wave. The extraction times used were 10 and 30 sec, and 1, 3, 5, 10 and 20 min. The horn-type system was set at initial temperature of 21 °C, and then raised to 24 °C after 1 minute, 60 °C after 15 minutes, and 63 °C after 20 minutes. Aglycone isoflavones increased during the first 5 min and then decreased after 15 min. The strong energy is demolished at the material surface and inside [Tatake et al., 2002]. Therefore, the ultrasonic energy and time need to be controlled. The contents of the glycosides isoflavones were abundant along with a large amount of aglycone isoflavones. Under the same conditions, the yields of isoflavones extracted by ultrasonic frequency at 20 KHz were 2.5-fold greater for glycosides and 3.3-fold greater for aglycones than those by dipping method. The ultrasonic method demonstrated a higher intensity and higher frequency than the dipping method.

CONCLUSIONS

The yields of isoflavones extracted from Korean soybean by ultrasonic waves with various conditions were compared with those obtained by dipping method. The effect on extraction efficiency of

variations in solvent composition, temperature, and extraction time was investigated. The experimental variables for ultrasonic extraction were frequency (20, 40, 68 and 110 KHz) and extraction time (10 and 30 sec, and 1, 3, 5, 10, 15, and 20 min). The effects of frequency on the extraction efficiency were almost negligible, but the efficiency increased with increasing extraction time. At an extraction time of 10 min, the yields of glycoside and aglycone isoflavones extracted by ultrasonic wave at 20 KHz were three-fold greater than those by dipping method. The extraction performance by ultrasonic energy was improved with excellent extraction efficiency of aglycones isoflavones being demonstrated with pure water as the extraction solvent at a frequency of 20 KHz.

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