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Preparative Separation of Isoflavones from Korean Soybean by HPLC

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Abstract: In this study, analytical and preparative HPLC systems were utilized to obtain isoflavones from Korean soybean. The mobile phases were the ternary system of water/acetonitrile/acetic acid. The optimum operating conditions were experimentally determined with the analytical column (C_{18}, 0.46×25 cm 5 micron and the preparative column packed with 15 micron. The experimental variables were the gradient conditions and mobile phase composition. The aglycones isoflavones of daidzein, glycitein and genistein were isolated and identified. The yield of solid extract was 5%, while the weight percentage of genistin, daidzein and genistein was 0.2 wt.%. It was found that the total amount of the aglycone isoflavones of daidzein and genistein extracted from the Korean soybean was 23.18 μg g^{-1}.

Key words: Soybean, solvent extraction, HPLC, isoflavones, daidzein, genistein

INTRODUCTION

Isoflavones are phytochemicals found in a variety of plants including soybeans that have been a popular Korean diet for centuries. As it contains 40% protein and 20% lipid with only a small amount of starch, soybeans are considered to be closer to the meat group rather than grain products. They may contribute to many of the health benefits attributed to soy foods (Peñas *et al*., 2004; Kim *et al*., 2005; Chang *et al*., 2004). Isoflavones, such as the glycosides of daidzin, glycitin and genistin, are quite abundant in soybeans. The chemical structure of the aglycones, aidzein and genistein, derived from these glycosides, have been frequently examined (Heinonen *et al*., 2003; Vendula *et al*., 2004). These isoflavones have several biological effects and are considered to be anti-carcinogenic. 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 and Shetty, 2004; Griffith and Collison, 2001). 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 and Ferreira, 2002; Choi *et al*., 2004). Typically, isoflavones had been extracted by water, Methanol (MeOH), Ethanol (EtOH), Acetonitrile (MeCN) and n-hexane solutions (Gaur *et al*., 2007). Achouri evaluated the extraction yield of isoflavones using three different solvents, methanol, ethanol and acetonitrile-HCl, with repeated extractions (Achouri *et al*., 2005). Also, several extraction methods for these compounds have been proposed, from classical soxhlet and stirring to using some new technologies, like nltrasound assisted extraction, supercritical fluid extraction and pressurized liquid extraction (Molamma and Conrad, 2006). The typically process technical was Solid-Phase Extraction (SPE) which is a simple preparation technique can be used to clean-up extracts as well as for extracting/concentrating soybean isoflavones from liquid food and its extracts (Mauricio *et al*., 2005). The structures of isoflavones are very similar to the human natural estrogens,

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which are hormones accessory for normal growth and development and to maintain good adult health, especially for women (Kim and Kwon, 2001; Bingham *et al*., 1998). In this study, a reversed-phase HPLC system was used to separate preparatively aglycones Korean soybean. The isoflavones of genistin, daidzein and genistein were identified.

MATERIALS AND METHODS

Chemicals

Sample was prepared by the standard chemicals (daidzin, genistin, daidzein and geinstein) of 2 mg was dissolved in ethanol then the concentration of the isoflavones was adjusted to 500 ppm. The standard chemicals of daidzein 99%, genistein 99%, daidzin 99% and genistin 99%, which were purchased from Sigma Co. (USA.). The HPLC-grade ethanol, methanol, acetonitrile and acetic acid were purchased from J.T. Baker (USA). Twice distilled water was filtered by pump (Division of Millipore, Waters, Milford, MA, USA), degassed with helium gas and filter (FH- 0.2 micron Waters, Milford, MA, USA). The Korean soybeans by Mejoo and Cellist Co. (Kangwon Do, South Korea) were extracted with 60% aqueous ethanol, then filtered through a filter (HA-0.45 micron, FH-0.5 μm) and membrane filter (0.2 micron Waters Co.)

Instrumentals

The experiments were performed with Waters HPLC 600E pump and 486 detector with Chromate 3.0 data acquisition system (Interface Eng.). Sufficient time was allowed for the stabilization of the column and detector signal after each injection and the solvents in reservoirs were continuously stripped with helium to degas the mobile phase. The wavelength was fixed at 254 um. The two different types of the columns used in this experiment were RS-tech (C_{18}, 0.46×25 cm, 5 micron and the empty staiuless steel columns (0.46×25 cm) in-house packed with 15 micron C_{18} (Lichrosphere, Merck). The flow rate was 1 mL min^{-1} and the injection volume was fixed at 20 μL. The ternary mobile phases were composed of water, acetonitrile and acetic acid. The Korean soybean was sieved at 32 micron, ground in a dry state for 2 min in a food mixer (Hannil Mixer FM-909T, 220W, 1.3A) and then underwent ultracentrifugation (Hannil Science Industrial, Model: Micro 17R Plus, South Korea). The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. Figure 1 shows the schematic diagram of extraction and purification of isoflavones from Korean soybean.

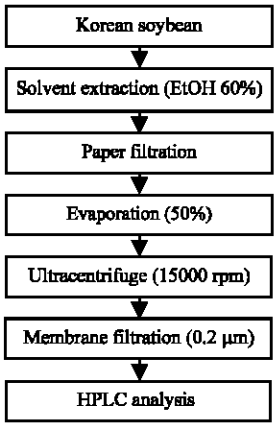

Fig. 1: Extraction and purification procedures

RESULTS AND DISCUSSION

To analyze the extract contained in the soybean, the commercially available C_{18} column was utilized. The mobile phases were composed of water with acetic acid and acetonitrile. Gradient mode was applied; the mobile phase composition of the reservoir A was 94.9/5.0/0.1 Vol.%, water/acetonitrile/acetic acid, while that of the reservoir B was 5.0/94.9/0.1 Vol.%. This mobile phase compositions in the reservoirs A and B were kept constant throughout in this work and the mobile phase composition of reservoir B was linearly increased from 0 to 30 vol.%. The calibration curves of the isoflavones were daidzin, genistin, daidzein and genistein and the correlation coefficient (r^2) was higher than 0.98 in each case.

As the composition of mobile phase was linearly changed and the content of acetonitrile was increased, the late-eluting components were eluted faster and showing good resolutions. In the column (0.46×25 cm) packed by 15 micron the amount of sample injected and concentration of Korean soybean concentrated extract were 20 μL and 1.69 g mL^{-1}, respectively. With the same mobile phase composition and gradient condition as that in the previous condition, in Korean soybean and all the standard chemicals were not commercially available, the precise quantitative analysis was not done. Also, this fragment requires revising. The content isoflavones was experimentally determined as 0.3%, where about 50% existed in the form of genistin. It was composed of glycoside (97%) and aglycones type (3%). The separation of isoflavones from Korean soybean was shown in Fig. 2.

Aglycones isoflavones of daidzein and genistein and glycoside isoflavones of 6"-o-malonylgenistin, daidzin, glycitin, genistin and 6"-o-acetylgenistin were contained. Those glycoside isoflavones were more included in the extract. At the same experimental conditions, the chromatogram of sample injected to a preparative chromatographic column was shown in Fig. 3.

The contents in ethanol extracts were changed and it was experimentally found that the extraction efficiency was the most favorable at aqueous solution of 60% ethanol. Korean soybean under these experimental conditions, the yield of solid extract was 5%, while the weight percentage of genistin,

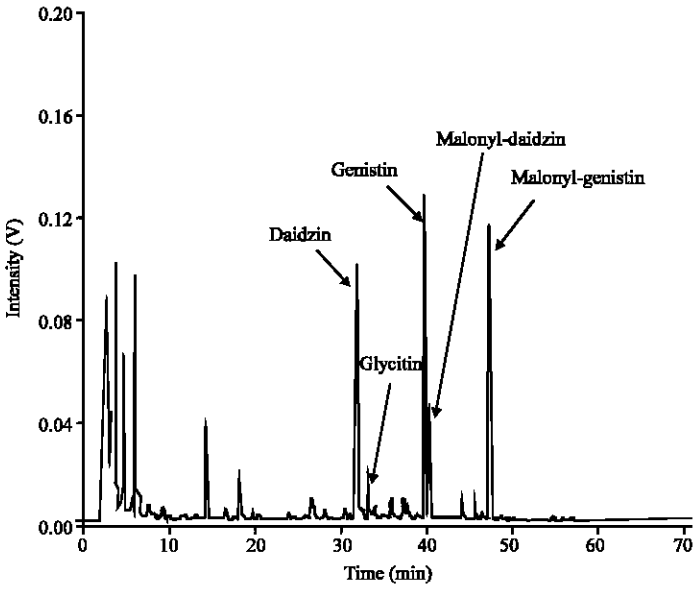

Fig. 2: Chromatogram of isoflavones from Korean soybean by analytical HPLC with 60% aqueous ethanol (RS-tech C_{18}, column, 0.46×25 cm, 5 micron)

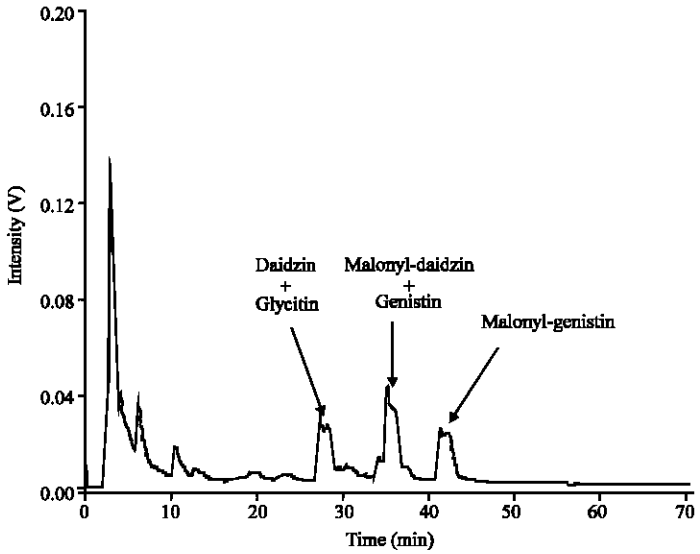

Fig. 3: Chromatogram of isoflavones from Korean soybean by preparative HPLC with 60% aqueous ethanol (Lichrosphere C_{18} column, 0.46×25 cm, 15 micron)

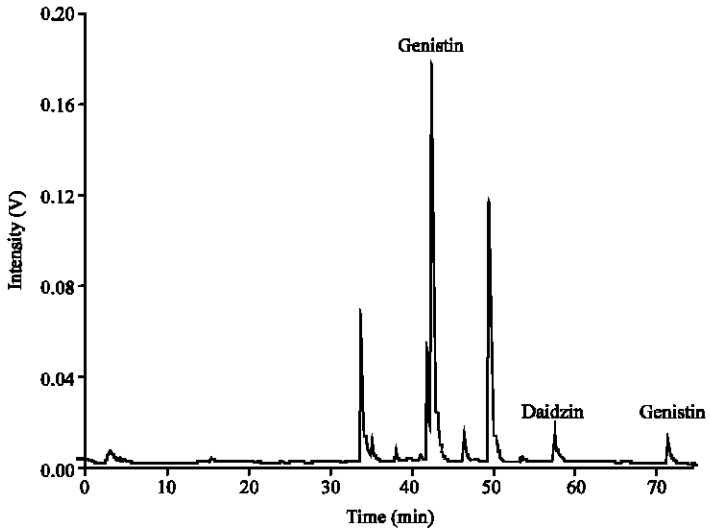

Fig. 4: Chromatogram of fractioned isoflavones from Korean soybean by analytical HPLC with 60% aqueous ethanol (RS-tech C_{18}, column, 0.46×25 cm, 5 micron)

daidzein and genistein was 0.2 wt.%. As might be expected, the resolutions of isoflavones were remarkably better and the retention times were slightly shorter in analytical column. Due to the increased column efficiency, the existences of aglycones isoflavones of genistein, daidzein and glycetein were experimentally confirmed. The effluents between 30 and 70 min in Fig. 3 were collected and they were analyzed by an analytical column and the results were shown in Fig. 4.

The optimum operating conditions were experimentally determined to analyze and collect the isoflavones from Korean soybean extract. The extraction efficiency was the most favorable at aqueous

solution of 60% ethanol. Korean soybean was purified by a preparative column with 15 μm packings and linear gradient elution mode. Under these experimental conditions, the yield of solid extract was 5%, while the weight percentage of genistin, daidzein and genistein was 0.2 wt.%. It was found that the total amount of the aglycone isoflavones of daidzein and genistein extracted from the Korean soybean was 23.18 $\mu g\ g^{-1}$. It is a significant fact the content of glucosides isoflavones in the soybean was remarkably higher than that in the Korean soybean.

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