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Determination of Daidzein and Genistein in Soy Milk in Iran by Using HPLC Analysis Method

S. GolKhoo, A.R. Ahmadi, P. Hanachi, F. Barantalab and M. Vaziri
Department of Biomedical Sciences, Woman Research Center, Alzahra University, Tehran, Iran

Abstract: The HPLC system separated completely isoflavonoids such as daidzein (15.2 min) and genistein (17.3 min). Initially, the concentrations of major isoflavone Genistein and Daidzein in the tested soy milk were determined. Commercial soy milk samples were analyzed for isoflavones and two major isoflavones detected: genistein $25.86 \text{ (mg L}^{-1}) \pm 0.66 \text{ SD}$ and daidzein $8.25 \text{ (mg L}^{-1}) \pm 1.13 \text{ SD}$. Concentrations of genistein in soy milk were higher than daidzein. The results obtained in this study can serve as a basis for estimating amount of soy milk can be consumed by people as related to its main isoflavone content.

Key words: Genistein, daidzein, isoflavones, soy milk, HPLC, phytoestrogens

INTRODUCTION

The health benefits of soybean products have been well documented. Epidemiological studies have indicated that the consumption of soybeans products may prevent certain cancers such as breast cancer, prostate cancer, colon cancer, (Alekel *et al.*, 1998; Anthony *et al.*, 1996), reduce the risk of hyperlipidemia, cardiovascular disease and osteoporosis (Arjmandi *et al.*, 1996; Barnes *et al.*, 1994), have a beneficial role in chronic renal disease (Fico *et al.*, 2000; Ranich *et al.*, 2001), lower plasma cholesterol (Franke *et al.*, 1995; Ho *et al.*, 2000), exhibit an antiatherosclerotic activity (Hillis and Isoi, 1965; Huff *et al.*, 1982) and decrease the risk of coronary heart disease (Lucas *et al.*, 2001). The commonly studied phytoestrogens are isoflavones (Fig. 1). Genistein and daidzein possess antioxidant, anticarcinogenic and anti-osteoporosis activities both *in vivo* and *in vitro*. The

intake of genistein and daidzein has been shown to provide protection against oxidative modification of Low-Density Lipoprotein (LDL) particles in human volunteers (Tikkanen *et al.*, 1998). When incubated in human plasma, some genistein and daidzein were incorporated into LDL particles (Kerry and Abbey, 1998) and incorporation of esterified genistein and daidzein increased resistance of LDL to copper mediated oxidation *in vitro* (Meng *et al.*, 1999). Consumption of soybean isoflavones was also found to be protective against DNA oxidation in human lymphocytes (Mitchell and Collins, 1999). In addition, genistein and daidzein also exhibited, *in vitro*, strong antioxidant potency in liposomes challenged with UV exposure, peroxy and hydroxyl free radicals (Record *et al.*, 1995; Tissier *et al.*, 2007). Isoflavones including daidzein and genistein are found almost exclusively in soybeans, which contain daidzein and genistein as the main glucosides. Thus, isoflavones are

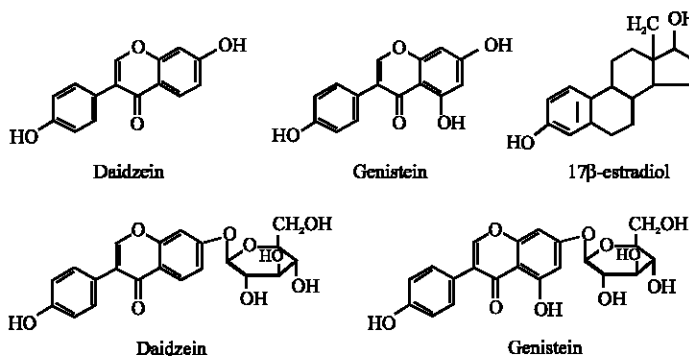


Fig. 1: Chemical structures of isoflavones and 17 β -estradiol

useful as food supplements for the purposes of enhancing human health and preventing the above diseases. Daidzein and genistein are hydrolyzed to daidzein and genistein, respectively, by β -glucosidase in the gastrointestinal system (Piskula *et al.*, 1999; Rucinska *et al.*, 2007; Atteritano *et al.*, 2008). However, recent investigations have shown that flavonoid glycosides, including genistein, phloretin and quercetin, can be partially absorbed without previous hydrolysis of glucose moieties (Paganga and Rice-Evans, 1997; Ho *et al.*, 2002). In addition, most of the genistein and daidzein is not present in form of aglycone but instead in the form of glucuronide and sulfate conjugates in blood (Hendrich, 2002). As shown in Fig. 1, they have a structure similar to the hormone estrogen and share some of its physiological properties. For these reasons, isoflavones are sometimes called phytoestrogens (Markham *et al.*, 1978; Piskula *et al.*, 1999). Therefore, isoflavones may not only have a variety of desirable physiological effects on the body, but they may also act as endocrine disruptors. Exposure to these phytoestrogens may pose a developmental hazard to infants, because soy products are becoming increasingly popular as infant foods. Though an accurate assessment of the evaluation of these contradictory health effects is difficult, knowing the existence and amount of isoflavones in foods is important. Even though there are a lot of publication exist regarding the concentrations of isoflavones in soymilk, since the product is not general in Persian diet. The aim of this study was to determine the concentrations of daidzein and genistein the major isoflavones of soy milk in Iran.

MATERIALS AND METHODS

This study, was carried out from 2006 to 2007 at the Women Research Center, Alzahra University, Iran.

Soy milk samples: Soymilk, supplied by MaxSoy Co. (Iran), The composition of this specific soy milk (as stated by manufacturer) was 12.5% protein, 1.25% Carbohydrates (2.5 g L^{-1} , sugars), 5.8% fat and 5% fiber, with pH 6.6. All samples used were of the same batch as stated by the manufacturer. All samples were stored at 4°C until use. Each soy milk samples were belong to different soy bean varieties, which are imported from Argentina to Iran.

Standards: Genistein and Daidzein were purchased from Sigma. Isoflavone standard solutions were prepared based on UV maximum absorbance and molar extinction coefficient (Ollis, 1962). The purity of the standards was

based on the percentage peak area according to the Beckman System Gold software and final concentrations were adjusted on the basis on these purities. Stock solutions were stored at -15°C and thoroughly warmed and redissolved at room temperature for 2 h to ensure complete solubility.

Isoflavone analysis: Isoflavones were extracted from soy milk samples by the addition of 8 mL of methanol to a 2 mL milk sample and shaking at 25°C for 2 h. Following incubation the mixture was transferred to centrifuge tubes and centrifugated for 20 min at 9000 rpm. The supernatant was then carefully filtered ($0.2 \mu\text{m}$) into Eppendorf tubes and analyzed by HPLC.

The HPLC system consists of Waters liquid chromatograph (Milford, MA, USA) equipped with a 600E multisolvent delivery system, an in-line degasser, a manual injection with 20 μL loop (Rheodyne 7125) and Waters 2487 dual λ absorbance detector. Empower[®] software was used for controlling the analytical system and data processing.

The liquid chromatographic method used for the determination of AA consisted of a gradient elution procedure with UV-Visible detection at 254 nm. Separations were carried out on a 5 μm RP C18 column of $250 \times 4.6 \text{ mm}$ (Spherical, Optimal[®] ODS-H, Capital HPLC, UK) fitted with a 5 μm RP C18 guard column of $20 \times 4.6 \text{ mm}$ (Spherical, Optimal[®] ODS-H, Capital HPLC, UK). The mobile phase employed was: A (80 mM tetra ethyl ammonium phosphate pH 2.5) and B (Acetonitril:A 70:30) of 0.5% NaH_2PO_4 that 50% A increased to 80% in 20 min. Flow rate of the mobile phase was 1.1 mL min^{-1} and an injection volume of 20 μL was used in quantitative analysis. The temperature of analytical column was kept constant at 25°C .

HPLC-grade methanol was purchased from Labscan Analytical Sciences (Bangkok, Thailand) and ammonium acetate from Sigma. All reagents used in isoflavone extraction and HPLC analysis were filtered through a $0.5 \mu\text{m}$ FH membrane (Millipore, Bedford, MA).

RESULTS AND DISCUSSION

The overall goal of this study was to evaluate the concentrations of isoflavones in soy milk. Analytes were routinely identified by retention times in various HPLC systems and by diode-array absorption patterns (Fig. 2-5). The HPLC system separated completely isoflavonoids such as daidzein (15.2 min) and genistein (17.3 min). Initially, the concentrations of major isoflavone Genistein and Daidzein in the tested soy milk were determined. Commercial soy milk samples were analyzed for

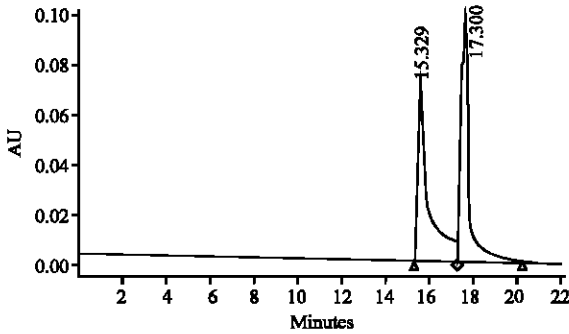


Fig. 2: HPLC Chromatogram of Genistein (RT= 17.3 min) and Daidzein (RT= 15.329 min) standard calibration curves

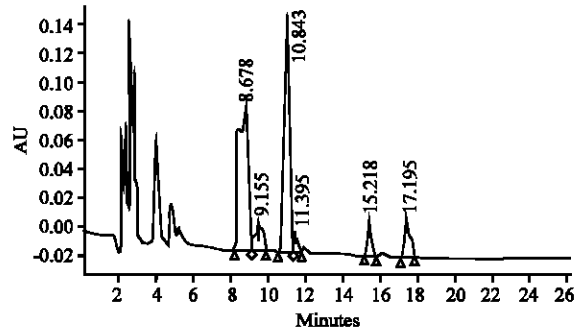


Fig. 5: HPLC Chromatogram of soy milk sample three, Genistein (RT = 17.195 min) and Daidzein (RT = 15.218 min)

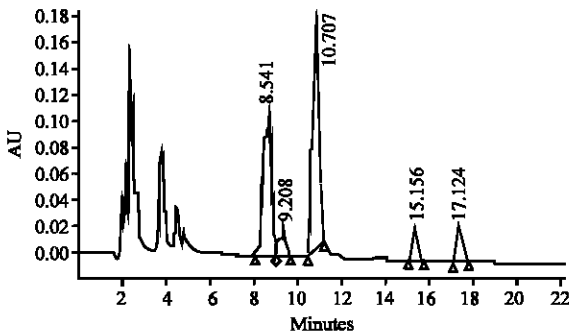


Fig. 3: HPLC Chromatogram of soy milk sample one, Genistein (RT = 17.124 min) and Daidzein (RT = 15.156 min)

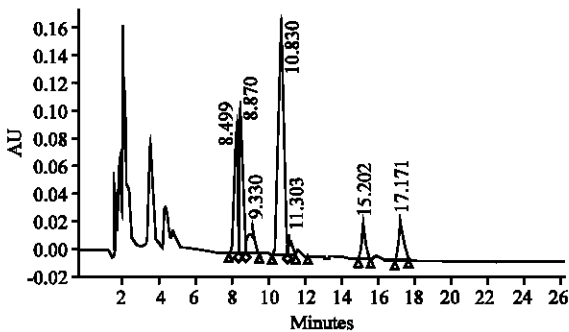


Fig. 4: HPLC Chromatogram of Soy Milk sample two, Genistein (RT = 17.171 min) and Daidzein (RT= 15.202 min)

isoflavones and two major isoflavones detected: genistein 25.86 (mg L⁻¹) ±0.66 SD and daidzein 8.25 (mg L⁻¹) ±1.13 SD. Concentrations of genistein in soy milk were higher than daidzein (Fig. 2-5). This profile of : genistein and daidzein derivatives is in agreement with earlier reports, showing that the genistein and daidzein concentrations in soy milk range from 24 to 130 mg L⁻¹

Table 1: Concentrations of genistein (RT = 17.3 min) and daidzein (RT = 15.2 min) on soy milk samples

Soy milk sample	Genistein (ppm)	Daidzein (ppm)
Sample 1	26.6	9.55
Sample 2	25.7	7.50
Sample 3	25.3	7.70

Genistein (mg L⁻¹) = 25.86±0.66, Daidzein (mg L⁻¹) = 8.25±1.13

and 12 to 94 mg L⁻¹, respectively (Choi *et al.*, 2000; Fukutake *et al.*, 1996; Wang *et al.*, 1990; Murphy *et al.*, 1999).

Table 1 shows the recoveries of 2 isoflavones in soymilk samples. Figure 2-5 show the HPLC Chromatogram of genistein and daidzein standard calibration curves and also HPLC Chromatogram of genistein and daidzein on three different soy milk samples. The recoveries for all the isoflavones ranged from 65 to 91%, which were a bit higher than those (60-90%) reported in the literature (Coward *et al.*, 1993; Murphy, 1981), probably because of difference in extraction solvents and methods used. In conclusion, the data indicate that there weren't significant differences between isoflavones content of three different soy milk samples. There results obtained in this study can serve as a basis for estimating amount of soy milk can be consumed by people as related to its main isoflavone content.

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