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Detection of Genistein in Soy Protein Isolate and Soy milk Powder by Spectrophotometric and Chromatographic Method

MD. ANISUR RAHMAN MAZUMDER*[1& 2], PARICHAT HONGSPRABHAS[2]

[1] Department of Food Technology and Rural Industries, Faculty of Agricultural Engineering and Technology, Bangladesh Agricultural University, Mymensingh- 2202, Bangladesh
[2] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, 50 Phahonyothin Road, Chatuchak, Bangkok, 10900, Thailand

Genistein proposed as a treatment for osteoporosis for postmenopausal women, elderly men, lowering cardiovascular disease and reduces hormone dependent cancers. Genistein also exerted inhibitory effect on lipid peroxidation induced in vitro by pro-oxidant agents on model and natural membranes on cultured cells and on low density lipoprotein. Genistein detection in soy products is very much important for Food Scientist. Gensitein can be detected by UV-Visible spectrophotometric and HPLC method. This study focused on the detection of genistein by HPLC and spectrophotometric methods. Genistein content of both soy protein isolate (SPI) and spray dried soy milk powder (SMP) was determined by spectrophotometry (93.12±1.15 and 74.78±0.75 mg/100g, respectively) were slightly higher but not significantly differ than HPLC analysis (89.67±5.16 and 72.34±0.27 mg/100g, respectively). This study suggested that genistein and its glycoside could be detected by spectrophotometric methods with high accuracy.

Key words: Genistein, spectrophotometry, HPLC, soy protein isolate, soy milk powder

Introduction

Phytoestrogens are naturally occurring plant compounds found in numerous fruits and vegetables. They are non-steroidal compounds that have estrogenic or/and antiestrogenic effects (Fatih, 2005) by sitting in and blocking receptor sites against estrogen receptors (ERs) α and β due to their structural similarity with estradiol. Phytoestrogens are categorized into four classes: the isoflavones (Sirtori *et al.,* 2005), stilbene (Cornwell *et al.,* 2004), lignans (Poluzzi *et al.,* 2014) and coumestans (Sirotkin *et al.,* 2014). Isoflavones are generally consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring. They are known as flavonoids, which are the largest and found in wide range of plant phenolics (Liu, 1997; Erickson, 1995). The soybean contains the highest amount of isoflavones, up to 3 mg/g dry weight (Liu, 1997; Rostagno *et al.,* 2004). The isoflavones have basically three types, with each type being present in four chemical forms. Isoflavones in soybean are mainly found as aglycones (genistein, daidzein, glycitein), β-glucosides (genistin, daidzin, glycitin), malonyl-β-glucosides (6′′-O-malonylgenistin, 6′′-O-malonyldaidzin, , 6′′-O-malonylglycitin) and acetyl-β-glucosides (6′′-O-acetylgenistin, 6′′-O-acetyldaidzin, 6′′-O-acetylglycitin) (Figure 1) (Mazumder and Hongsprabhas, 2016; Lee and Lee, 2009). Aglycones are flavonoid molecules without attached sugars or other derivatives. Aglycones are especially important among other isoflavone because they are readily bioavailable to humans (Lee and Lee, 2009). β-glucosides may also carry additional small molecular modifiers, such as malonyl and acetyl groups. Sugar-linked flavonoids are called glucosides due to their glucose linkage to flavonoids (Mazumder, 2016).

Isoflavones are non-nutrients, because they neither yield any energy nor function as vitamins. Nonetheless, isoflavones have received considerable attention nowadays as phytoestrogens. To date, isoflavones have received attentions as to play significant roles in the prevention of several diseases and they are considered as health-promoting substances (Mazumder and Begum, 2016). This is because of their weak estrogenic property and other beneficial functions (Rahman Mazumder and Hongsprabhas, 2016; Bedell *et al.,* 2014; Rietjens *et al.,* 2013). Researches revealed that soy isoflavones and their glycosides are associated with a lowering of cardiovascular disease (Adlercreutz, 1990), hormone-dependent breast and prostate cancers (Yu *et al.,* 1991), colon cancer (Rose *et al.,* 1986), menopausal symptoms (Clarkson, 2000), osteoporosis (Adlercreutz, 1992), atherosclerosis (Armstrong and Doll, 1975) and improve the arterial elasticity in menopausal women, similar to hormone replacement (Wilcox and Blumenthal, 1995).

In 1999, the US Food and Drug Administration approved a health claims for the cholesterol-lowering effects of soy protein, largely based on a meta-analysis of 38 clinical trials that reported significant decreases in total and low-density lipoprotein (LDL) cholesterol and triglycerides with soy protein intake (25 g/day) compared with animal protein consumption (Adlercreutz *et al.,* 1995). Therefore, genistein can be accounted as the main isoflavone responsible for the biological activity of soy extracts.

Genistein proposed as a treatment for osteoporosis for postmenopausal women, elderly men (Yamaguchi and Goa, 1998) lowering cardiovascular disease (Rahman Mazumder and Hongsprabhas, 2016) and reduces hormone dependent cancers (Rahman Mazumder and Hongsprabhas, 2016).

*Corresponding author, email address: anis_engg@bau.edu.bd

However, genistein could also act as an antioxidant in *in vivo* and *in vitro* (Mazumder *et al.,* 2019; Rahman Mazumder and Hongsprabhas, 2016). The in vivo bioavailability experiment for genistein and its glycoside genistin showed that genistein is readily bioavailable, being observed in portal vein plasma at the first point of detection at 15 min after dosing. The results indicated that the bioavailability of genistein was higher for the aglycon than for its glycoside (Rahman Mazumder & Hongsprabhas, 2016; Monteiro *et al.,* 2004).

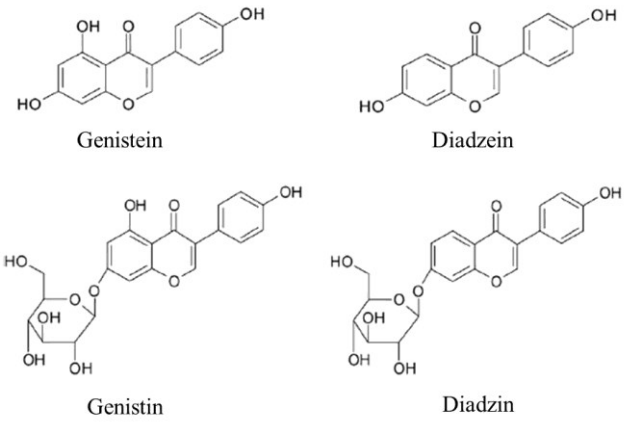

Figure 1. Chemical structure of genistein, diadzein, genistin and diadzin.

Genistein can be analyzed by chromatography and spectrophotometric methods (César *et al.,* 2008). The assay of flavonoids results complex formation with aluminum chloride ($AlCl_3$) is the main principle for UV absorbance determination of gensitein. Flavonoids containing 5-hydroxy-4-keto, 3-hydroxy-4-keto or *o*-dihydroxyl systems are able to chelate with $AlCl_3$ and the reaction is disclosed by a bathochromic shift of the bands in the UV-Visible spectrum (César *et al.,* 2008; Harbone *et al.,* 1975; Mabry *et al.,* 1970). Genistein and its glycoside, genistin, present in the soy products only can react with $AlCl_3$. Spectrophotometric method is quicker and less expensive than HPLC method. Detection of genistein and its glycoside by HPLC need more than 7 hours whereas spectrophotometirc method need around 4 hours. This study focused on the comparison between HPLC and spectrophotometric method for genistein detection to find whether spectrophotometric method is valid or not valid for genistein detection.

Materials and Methods

Chemicals

Soy protein isolates (94.40% protein and 5.40% moisture) supplied by Vicchi Enterprise Co. Ltd., Bangkok, Thailand), spray dried soymilk powder (1.98% moisture, 57.26% protein and 27.60% fat), genistein (Synthetic; analytical grade, Product code G6649, Sigma-Aldrich, St. Louis, USA), methanol (CH_3OH; HPLC-grade; J.T. Baker; Noisy le Sec, France), acetonitrile (CH_3CH; HPLC-grade; J.T. Baker; Noisy le Sec, France), trifluoroacetic acid (CF_3COOH; HPLC-grade; J.T. Baker; Noisy le Sec, France), hexane ($C_6H_{14;}$ analytical grade; Mallinckrodt Chemicals; USA), de-Ionized water (H_2O; HPLC-grade; RCI Labscan, Thailand), ethanol (C_2H_5OH; analytical grade, MERCK, Merck KGaA, Darmstadt, Germany) and aluminum chloride ($AlCl_3$; analytical reagent grade, Sigma – Aldrich, St. Louis, MO, USA) were used in analysis.

Spectrophotometric methods

Sample preparation and extraction

Extraction of genistein from foodstuffs and their qualitative and quantitative were performed as previously reported by César *et al.* (2008) with some modification. Briefly, 25 mg of dry sample were accurately weighed in a 50 mL volumetric flask. The sample was previously dried at 105°C for 4 h with an air oven dryer (MembartGmBH+ Co., Büchenbach, Germany). A 20 mL of 99.9% methanol was added to the flaskand the solution was sonicated (UC-10200B, AT0100191, Ultrasonic processor, Chrom Tech, Taipei 104, Taiwan) for 20 min. The volume was adjusted to 50 mL with 99.9% methanol and filtered through 0.2 µm membrane filter (0.2 µm FG, Fluoropore^{TM}Membrane Filters, FGLP02500, R2PA14822, Ireland). An aliquot (5 mL) was transferred to a 25 mL volumetric flask and 1 mL of $AlCl_3$ solution (2% w/v in 99.9% methanol) was added. The flask volume was made up 25 mL with 99.9% methanol. Similarly, blank solutions for sample were also prepared without the addition of $AlCl_3$.

Preparation of genistein standard solution and detection

5 mg of genistein were accurately weighed in a 50 mL volumetric flask. 30 mL of 99.9% methanol was added to flask and sonicated (UC-10200B, AT0100191, Ultrasonic processor, Chrom Tech, Taipei 104, Taiwan) for 20 min. The volume was adjusted to 50 mL with 99.9% methanol. An aliquot of 5 mL was transferred to a 25 mL of volumetric flask. 1 mL of $AlCl_3$ solution (2% w/v in 99.9% methanol) was added to the flask. The flask volume was completed 25 mL with 99.9% methanol. Stock solution was diluted in 99.9% methanol to 1.5, 6, 18, 30, 42 and 54 µg/mL. 1 mL of 2% (w/v) $AlCl_3$ was added to each diluted solution. A calibration curve for concentration *vs* absorbance was plotted and the obtained data were subjected to regression analysis using the Least Square Method. Similarly, blank solutions for genistein standard sample were also prepared without the addition of $AlCl_3$.

UV-Visible spectrophotometric analyses were carried out on a Microplate reader (TECAN infinite 200, Tecan Austria GmbH, 5082 Grödig, Austria). UV-Visible spectrum of standard genistein solution and samples were observed in the range of 200 to 500 nm after reaction with $AlCl_3$. However, 382 nm wavelengths were defined for the quantitation of genistein and genistin in soy products (Cesar *et al.,* 2008). Spectrophotometric determination of soy dry extract solutions was carried out immediately after the $AlCl_3$ addition and at regular intervals of 10 min, until 240 min.

HPLC method

Sample extraction

Extraction of genistein from food stuffs and their qualification and quantification were performed as previously reported by Coward *et al.* (1993); Fukutake *et al.* (1996) with some modification. At first, samples (5 g) were extracted with 50 mL of 80% methanol by sonication (UC-

10200B, AT0100191, Ultrasonic processor, Chrom Tech, Taipei 104, Taiwan) for 30 min at 45°C. These extract solutions were centrifuged at 2500 g for 20 min and the supernatants were evaporated to dryness using a centrivap evaporator (Labconco, Kansas city, MO, USA). The dried extracts were dissolved in 5 mL of 50% methanol. Liquid-liquid extraction by 3 times of 20 mL of n-hexane was carried out to remove lipids from the samples. After evaporation of the aqueous methanol phase, the residue was dissolved in 2.5 mL of 80% methanol and an aliquot was filtered through 0.2 µm membrane filter (0.2 µm FG, Fluoropore™Membrane Filters, FGLP02500, R2PA14822, Ireland) before analysis by HPLC.

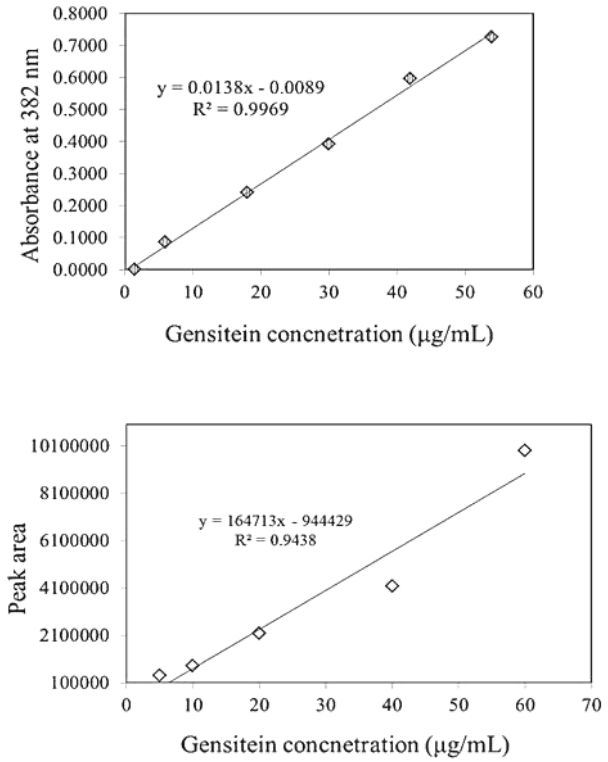

Figure 2. Standard curves for genistein determination for UV spectra (top) and HPLC (bottom) analysis.

HPLC analysis for genistein

Acetonitrile and trifluoroacetc acid were filtered separately through 0.45 µm polytetrafluoroethylene (PTFE) and de-gassed in an ultrasonic bath for 30 min before use. Six standard concentrations of pure (≥98%) genistein were prepared. They were: 0.1 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL and 40 µg/mL in 99.9% ethanol. Injection volume was 20 µL, column used was octadecylsilane (ODS) column (5 µm particle size, 4.6 x 250 mm) and a mobile phase of a gradient of 0 – 60% acetonitrile in 0.1% trifluoroacetic acid was pumped in at a flow rate of 1.2 mL/min at 25°C. Genistein was monitored by UV absorbance at 262 nm wavelength. The amount of genistein were estimated from standard curves. The retention time for genistein was 60 min.

Data analysis

Each experiment was carried out in three separate trials. The data were analyzed by analysis of variance (ANOVA) or t-test where appropriate with significance at $p<0.05$ and Duncan's multiple range tests. All statistical analyses were performed using SPSS software Version 17.

Results and Discussion

The UV-Visible spectrums were recorded for a both gensitein-$AlCl_3$ and genistein blank solution. The result showed an intense absorption band at 382 nm for gensitein-$AlCl_3$, which was not found in the genistein alone, the result was similar with result found by César *et al.* (2008). This absorption band is the indicator of complex formation between genistein-$AlCl_3$. A similar kind of absorption band was also found in the UV-Visible spectrum of soy protein isolate (SPI) and spray dried soymilk powder (SMP) after reaction with $AlCl_3$. The time interval for gensitein-$AlCl_3$ complex was evaluated for spectrophotometric detection. The absorbance of genistein- $AlCl_3$ complex reaches its maximum after 5 min of reaction and its stable for 240 min, since there was no significant variation in absorption was detected during this period. Based on these findings, after $AlCl_3$ addition, a period of 10 min was defined as the optimal detection time for quantitation of genistein in SPI and SPM. Similar result was found by César *et al.* (2008), they found a period of 10 min after $AlCl_3$ addition was defined as the optimal detection time for quantitation of genistein and genistin in soy dry extract samples.

For spectrophotometric method, plot of absorbance *vs* genistein solution showed a linear correlation (R^2= 0.9969) after reaction with $AlCl_3$ (Figure, top 2). The significance of the intercept obtained in the calibration curve was tested and this parameter was not statistically significant ($P>0.05$). The genistein content of SPI and SMP were calculated using this graph. For HPLC, plotting peak area *vs* time showed a linear correlation (R^2= 0.9438) (Figure 2, bottom) and this graph was used to calculate the genistein content of SPI and SMP.

Table 1

Genistein content in soy protein isolate (SPI) and spray dried soymilk powder (SPM) obtained by spectrophotometric and chromatographic methods.

Sample	Amount of genistein per 100g sample (mg/100g)		USDA (1999) data for genistein (mg/100g)
SPM	93.12 ± 1.15[a]	89.67[a] ± 5.16	78.90
SPI	74.78 ± 0.78[a]	72.34[a] ± 0.27	59.62

Means in the same row followed by different superscripts are significantly different ($P > 0.05$).

Most of the commercial soy products contained 53 to 65% gensitein of total isoflavones. The results obtained by the spectrophotometric method correspond to both genistein and genistin contents, expressed as genistein. In the HPLC analysis, the results are expressed as genistein content after hydrolysis. Amount of genistein determined by spectrophotometry method was not significantly differ from the amount assayed by the HPLC method ($P>0.05$) although, there was a variation in sample preparations including

solvents and temperature between both spectrophotometry and HPLC methods. Geisntein concentration in SPM and SPI showed in Table 1. Interestingly, the isoflavone content of SPI is much lower than that SPM because the mild alkali extraction used in the production of SPI causes isoflavone losses of approximately 53% (Wang and Murphy, 1996). However, genistein content of both SPM and SPI was found higher in spectrophotometric and HPLC method than USDA data (Table 1).

Conclusions

UV-Visible spectrophotometric detection of genistein was found to be simple and suitable technique for soy products. The spectrophotometric method is cost and time efficient, than the HPLC method. Hence, it can be applied for pharmacies and laboratories to detect the genistein or may be used as a preliminary assay to evaluate the genistein content in soy products. However, HPLC is used for more precise and accurate detection of genistein.

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