

Identification and quantification of isoflavones in Bangladeshi soy-milk, masoor and mung dals

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Abstract. The present study was carried out to identify and quantify of the isoflavones in soybean, masoor dal, mung dal produced in Bangladesh. An amount of 350 ml soy-milk was prepared from 100 g powdered bean following standard procedure and kept in a refrigerator. The milk was then kept in four flasks, frozen in a methanol freezer, and dried into powder with a freeze-dryer. The dried soy-milk, masoor dal and mung dal powders were refluxed with n-hexane in boiling water to remove oil/fatty materials from it, and ethyl acetate was used for extracting the oil-free powder. The ethyl acetate extract of these three foods was dried completely, re-dissolved in a definite amount of acetonitrile (ACN), and analyzed by HPLC-PDA on C18 column using mobile phase, ACN-H₂O (75:25; flow rate: 0.5 ml/min; wavelength: 268 nm, loop size: 20 µm, and running time: 10 min). Genistein and daidzein were identified in the oil-free sample extracts by comparing the retention time of the certified standard genistein and daidzein, purchased from Sigma-Aldrich. The quantification of isoflavones was done using the external calibration curve of the two certified samples, which was linear (r^2 for genistein and daidzein was 0.999 and 0.997 respectively). LOD (S/N ratio-3:1) and LOQ (S/N ratio-10:1) were, respectively, 0.0045 and 0.0135 ppm and 0.25 and 0.75 ppm, in genistein and daidzein. Daidzein and genistein were identified in the locally-produced soybean, masoor dal, and mung dal, and the number of total isoflavones in the foods were within the acceptable range.

Keywords: Isoflavones, daidzein, genistein, HPLC-PDA, soy-milk, masoor dal, mung dal, Bangladesh.

INTRODUCTION

Phytoestrogens, the naturally-occurring chemical elements of plants, have estrogenic, anti-estrogenic or anti-androgenic effects in animals and human-beings. They are classified into three groups, that is, isoflavones, coumestans, and lignans (Mazur and Adlercreutz, 1998). Their major sources in human diets are isoflavones, which are particularly abundant in soybeans and soy products. They are also found in legumes (Mazur *et al.*, 1998), seeds (Liggins *et al.*, 1998), vegetables (Liggins *et al.*, 2000a), fruits (Liggins *et al.*, 2000b), and other types

of plants, such as unripe fruits (pericarps), seedlings (leaves), flowers (Kato *et al.*, 1992), and red clover (Wu *et al.*, 2003). Isoflavones are produced almost exclusively by the members of the Leguminosae (bean) family. They include genistein and daidzein and have several features in common with estradiol-17β (Song *et al.*, 1998), the most potent mammalian estrogen. Daidzein and genistein are also found naturally in the form of glycoside and aglycones. Figure 1 shows the structure of the genistein, daidzein, and estradiol-17β. A growing number of scientific

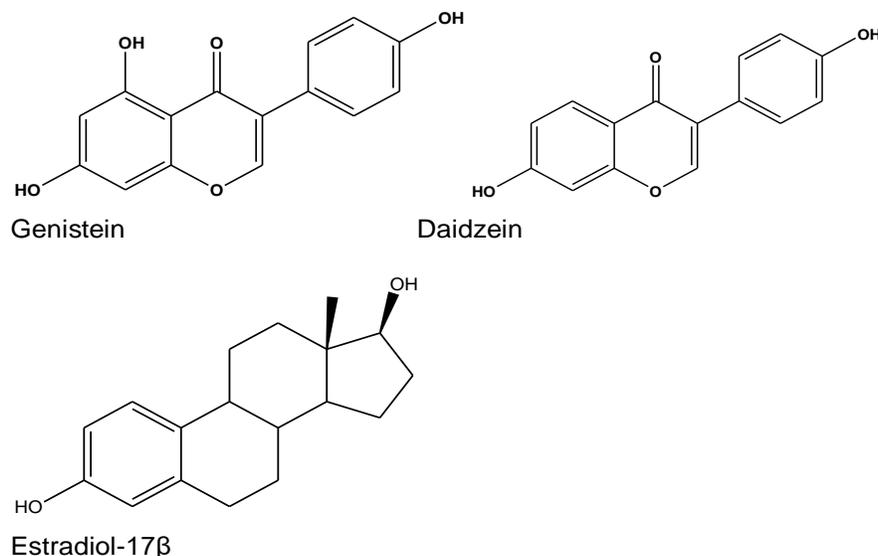


Figure 1. Structure of genistein, daidzein and estradiol.

works are now available on the beneficial effects of isoflavones-rich foods on health, which may be due to their weak estrogenic activities.

Results of epidemiological studies suggest that the consumption of soybeans and soy foods is associated with lower risks for several types of cancers, including breast, prostate, and colon (Messina, 1995; Messina *et al.*, 1997), cardiovascular diseases (Anderson *et al.*, 1995), and bone health (Nurmi *et al.*, 2002).

Soy-milk and tofu are the two most popularly-consumed soy foods in the East Asian region and in some countries of the Southeast Asian region. They are commonly served in food courts and supermarkets. Their consumption continues to increase since people are becoming more health conscious. In those regions, few studies (Tepavčević *et al.*, 2011; Wang and Murphy, 1994; Klump *et al.*, 2001; Prabhakaran *et al.*, 2005; Khoo and Ahmad, 2008) were conducted on the quantification of isoflavones in soybean, soy foods, and some fruits.

Soybean and soy-milk are not popular in Bangladesh. Although soybean is now being cultivated in some places of Bangladesh, it is not yet available in the local market. Our interest in isoflavones derives from their health-protective properties. At present, there is an increasing demand for soybeans in Bangladesh, along with the growing public knowledge on the benefits of the soy-rich foods.

On the contrary, other members of the Leguminosae family such as lentils (dal) are common food items among the people of Bangladesh, and these have been grown here for a long time. In the case of short supply, masoor dal and mung dal (green gram) are imported to meet the need of protein source and they also contain isoflavones. Despite this, mung and masoor dals have not been well-studied for their isoflavones content, except the one study (Mazur *et al.*, 1998). No data on the amount of isoflavones in soybean and mung and masoor dals

produced in Bangladesh are available. Only few studies (Saleh *et al.*, 2011; Bhuiyan, 2013; Bhuiyan *et al.*, 2017) were conducted to explore the intake of isoflavones and their beneficial effects in postmenopausal women. The authors of the above studies reported, as a limitation, that they calculated the content of isoflavones based on literature which is not authentic due to the variations in the quantity of isoflavones in different countries.

To the best of our knowledge, no studies have been undertaken to identify and also to quantify the content of isoflavones in soybean and non-soy foods, such as mung dal and masoor dal, produced in Bangladesh, which prompted us to conduct this study. The objective of the present study was to identify the presence of isoflavones and also to quantify isoflavones in soybean [*Glycine max* (L.) Merr.], mung [*Vigna radiata* (L.) R. Wilczek] and masoor dals [*Lens culinaris* (Medik.)] produced in Bangladesh.

MATERIALS AND METHODS

Samples

Samples of soybean seeds were collected from Jessore, the southern agricultural district of Bangladesh. Samples of mung dal and masoor dal were purchased from a local supermarket of Dhaka city. The dry soybean, mung dal, and masoor dal were dried again, ground into powder (200 mesh) with a laboratory grinder machine, and kept in three separate air-tight containers in a refrigerator until analyzed.

Chemicals

The authentic standards of daidzein and genistein were

purchased from Sigma-Aldrich and were preserved at a temperature of 4°C and at -20°C respectively. Liquid chromatography (LC) grade acetonitrile (ACN) and acetone were purchased from Merck, Germany. Extra pure analytical grade ethyl acetate (EtOAc), n-hexane, and anhydrous sodium sulfate (Na₂SO₄) were purchased from Merck KGaA (Darmstadt, Germany). Deionized and hydrocarbon-free water, used for analysis, was made from a water purification system (BOECO, Germany).

Preparation of soy-milk

The whole amount of soybean (100 g) was immersed in drinking-water in a pot for 4 to 5 h. The soft beans (water soaked) were washed with water, blended into mould using a kitchen blender. An amount of 500 ml water was added to the mould, boiled for three minutes, and stirred with a wooden kitchen stirrer. The milk was collected by squeezing through a pre-cleaned cloth filter. The prepared milk was boiled again for 20 min and stirred to reduce its volume to ~350 ml. The milk was then cooled and kept in a refrigerator before use.

The prepared milk was transferred to four volumetric ground joint flasks (500 ml each), frozen in a methanol freezer, and dried into powder with a freeze-dryer (Hetosicc CD 52 Heto Lab Equipment, Denmark).

Extraction of isoflavones from soy-milk, masoor dal, and mung dal

The isoflavones were extracted following the method described by Peñalvo *et al.* (2004) with slight modification and validation. Seven grams of soy-milk powder was weighted and transferred to a round-bottomed flask and refluxed using n-hexane (200 ml × 3; 30 min) in boiling water bath. The extract was filtered and discarded. The oil-free soy-milk powder was dried at room temperature. The hexane extract-free residue was further extracted using ethyl acetate (100 ml × 3; boiling water bath; 30 min) and filtered. The ethyl acetate extract was treated with anhydrous magnesium sulfate and filtered, and the filtrated substance was evaporated into dry mass using a rotary vacuum evaporator (Büchi; No. 517-6100-00) and re-dissolved in LC grade ACN. The residue was discarded (Figure 2).

The same method was followed for the extraction of masoor dal and mung dal (Figure 3). The samples of masoor dal and mung dal were dried in an oven at a temperature of 105°C and ground into powder using a locally-made grinder machine. The dried powder (25 g) was extracted using n-hexane (200 ml × 1; 30 min) in the refluxing condition. The samples were filtered by decantation followed by filtration in vacuum. The organic phase was discarded, and the residues were dried at room temperature. The hexane extract-free residues

were further extracted with ethyl acetate (100 ml × 3; boiling water bath; 30 min) and filtered. The ethyl acetate extracts were treated with anhydrous magnesium sulfate and filtered, and the filtrated samples were evaporated to dry mass using a rotary vacuum evaporator (Büchi; No. 517-6100-00) and re-dissolved in LC grade ACN. The residue was discarded.

All the extracts (soy-milk, masoor and mung dals) in acetonitrile were filtered using a LC sample filter having a pore size of 0.22 µm [PTFE (polytetrafluoro ethylene)] syringe filter cartridge and transferred to a sample vial of 2 ml size, and analysis was done by HPLC-PDA.

All the secondary standard and working standard solutions were prepared from the primary standard solutions of daidzein or genistein. The primary standard solutions (200 µg/g) were prepared by dissolving separately-certified daidzein (0.002 g) and genistein (0.002 g) in LC grade ACN (10 ml). The prepared solutions were labeled indicating the name of the standard, concentration, solvent, and the dates of preparation. The meniscuses of the solutions were marked with permanent ink. These primary standard solutions were stored in a freezer at a temperature of -20°C.

Analysis by HPLC-PDA

For all the chromatographic analyses, a Shimadzu SCL 10A *vp* LC system (Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD-10A *vp*) having a high-pressure binary pump was attached. A Rheodyne injector (loop size 20 µl) column at ambient temperature was used for analysis.

High-performance liquid chromatographic (HPLC) analyses were performed on a Supelco discovery reversed phase C18 column (25 cm × 4.6 mm *i.d.* particle size: 5 µm). Standards and cleaned extracts (100 µl) were injected through a Rheodyne injector. Separations were carried at 268 nm using an isocratic mobile phase of acetonitrile and water (ACN: H₂O) (75: 25), with a flow rate of 0.5 ml/min, and the running time was 10 minutes. The HPLC system was conditioned by passing mobile phase until the smooth baseline was obtained. The certified standards of daidzein and genistein (10 µg/ml) were injected separately, and the retention time of the two isoflavones was found to be 5.52 and 6.03 min respectively before the analysis of soy-milk, masoor dal and mung dal extracts by HPLC-PDA. The clean extracts of soy-milk, masoor dal, and mung dal were injected, and two peaks were found to be at 5.5 and 5.9 minutes, respectively, for soy-milk and masoor dal and mung dal. Daidzein and genistein were found to be present in the soy-milk powder and in the masoor dal and mung dal powder.

The presence of daidzein and genistein in the three different sample foods was identified in the same

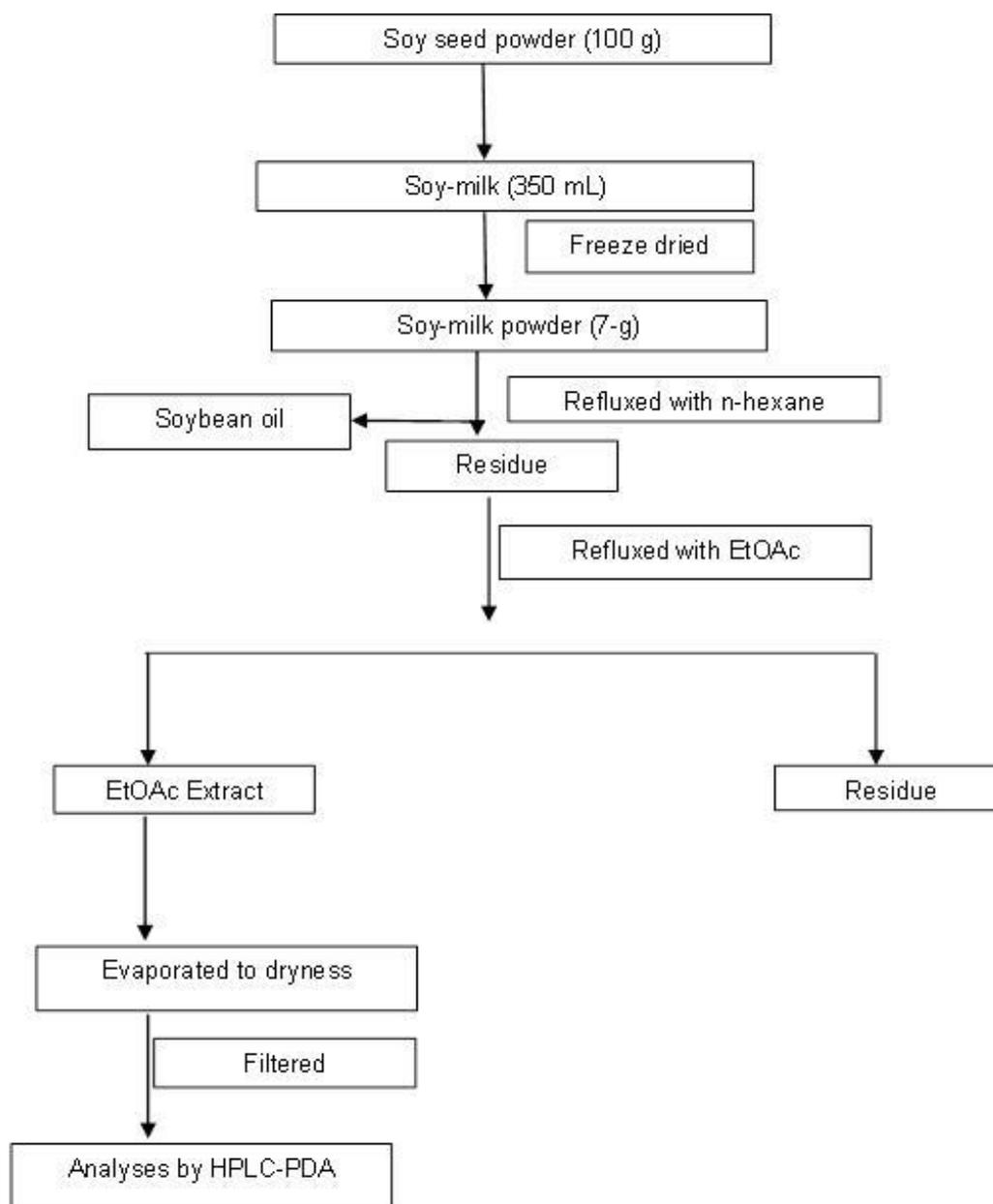


Figure 2. Extraction of isoflavones from soy-milk

retention time using HPLC-PDA. However, for further confirmation, only the cleaned soy-milk powder extract was also analyzed by liquid chromatography-mass spectrometry using Shimadzu LCMS-8050 with electrospray ionization (ESI), a triple quadrupole (QQQ) mass analyzer. Nebulizing and collision gas was N_2 . Separations were performed on a Shim-pack GISS C_{18} column (250 × 4.6 mm i.d.; particle size 5 μ m). The size of the carrier gas pipe was 5 m. The molecular mass of daidzein was 254.2 $g\ mol^{-1}$. Multiple-reaction monitoring (MRM) measurement was conducted by ESI using positive mode. The major fragment ions were observed at 186, 253 and 339, respectively.

Quantification of isoflavones

The quantitation of daidzein and genistein in soy-milk, masoor dal, and mung dal was done with respect to the external calibration curve of daidzein and genistein. The standard solutions of daidzein and genistein at the concentrations of 5, 10, 15, 20, and 25 μ g/ml were injected into HPLC-PDA. Two calibration curves were made from above five solutions of the daidzein and genistein by plotting area vs concentration (μ g/g).

For quantification, the concentration of the corresponding analyte was obtained from the standard calibration curve taking into consideration that the peak

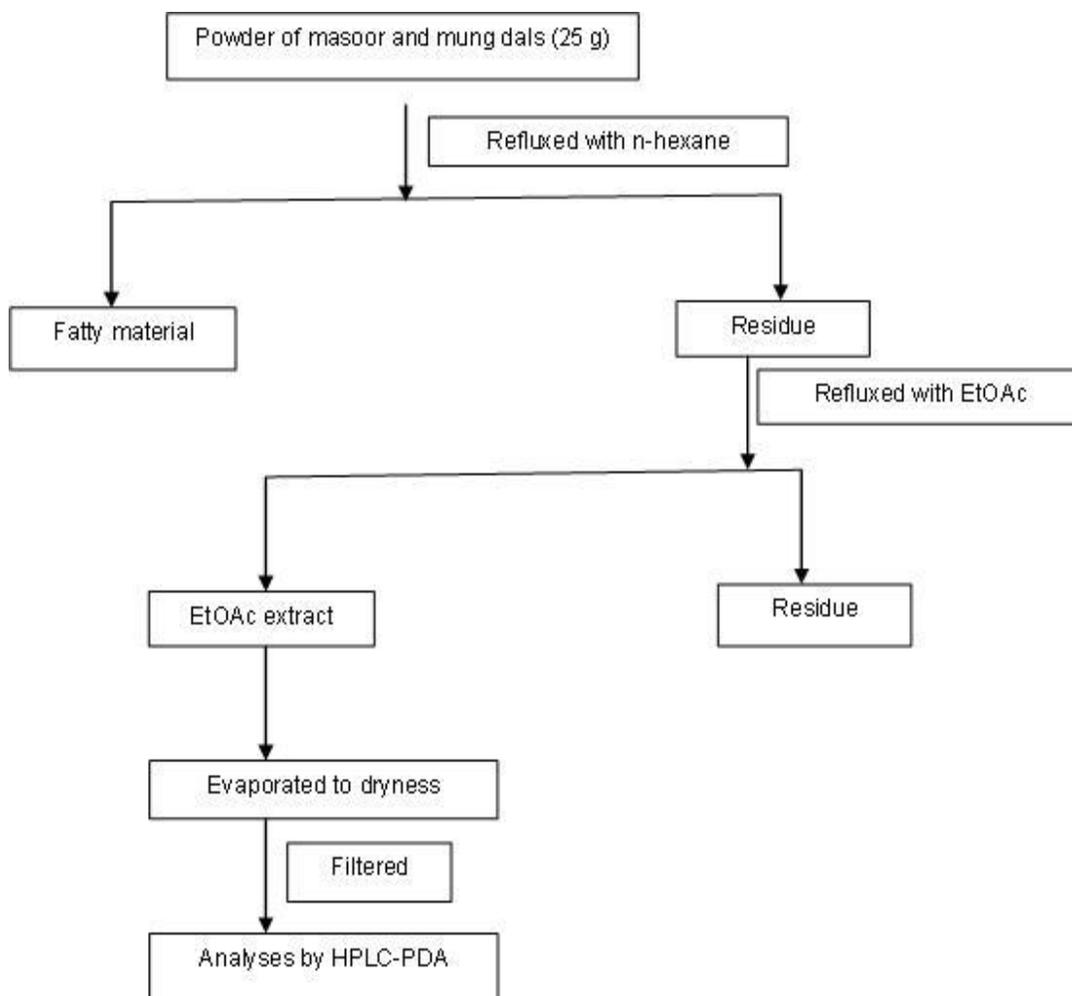


Figure 3. Extraction of isoflavones from masoor and mung dals powder

area was in the midpoint of the curve (considering linearity of the curve). A number of unknown analytes in the respective samples were identified using the following formula:

Amount of unknown sample

$$= \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Area}_{\text{Std}} \times \text{Conc.}_{\text{Matrix}}}$$

Recovery experiments

Three grams soy-milk powder was taken in a Teflon tube, and the standard daidzein (10 µg/ml) was added to the powder, shaken for 30 s, and kept at room temperature for two hours. The spiked and un-spiked (control) samples were extracted following the same extraction procedure as was done for soy-milk powder. The

following formula was used for recovery experiments:

$$R = \frac{A_m \times C_{st}}{A_{st} \times C_m} \times \frac{100}{M_{st}}$$

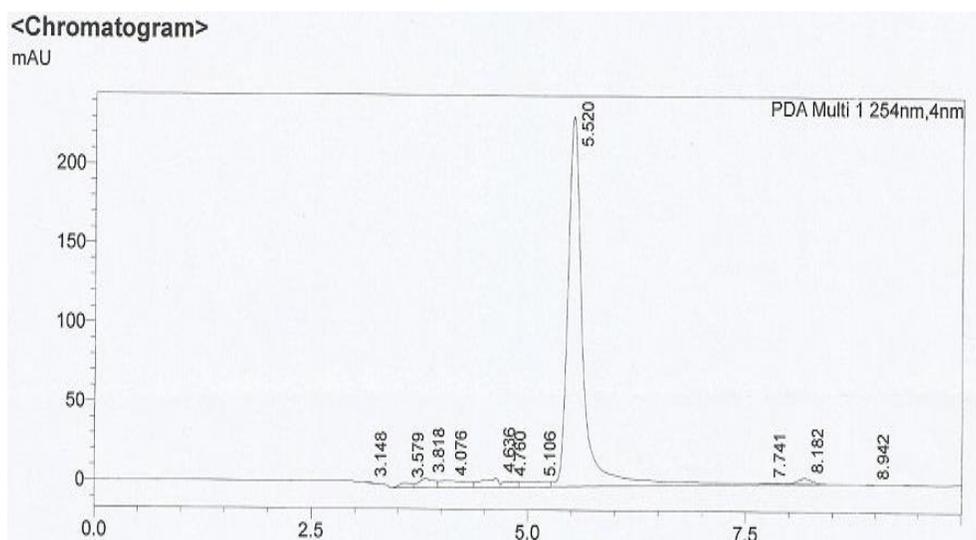
Where R is the recovery (%), A_m is the peak area of the analyte in the matrix, A_{st} is the peak area of the analyte in the standard, C_m is the concentration of the analyte in the matrix, C_{st} is the concentration of the analyte in the standard, and M_{st} is the mass of the analyte in the standard.

RESULTS AND DISCUSSION

Soybean is one of the important sources of edible oil, and several soy products such as soy-milk, soy-biscuits, tofu, etc, are used as food. In the study, soy-milk was collected to identify and quantify the presence of isoflavones in it. Oil was removed before the extraction of

Table 1. Linearity, limit of detection (LOD), and limit of quantification (LOQ).

Isoflavones analyzed	λ (nm)	r^2	LOQ ($\mu\text{g/ml}$)	LOD ($\mu\text{g/ml}$)
Genistein	268	0.999	0.0135	0.0045
Daidzein	268	0.997	0.75	0.25

**Figure 4.** Chromatogram of daidzein.

isoflavones for avoiding possible binding the oil with the non-polar stationary phase of C18 LC and also to have less matrix effect. Isoflavones were identified and quantified in oil-free soy-milk for method verification in the HPLC-PDA system with respect to certified standard isoflavones. Subsequently, the same method was followed for masoor dal and mung dal. The quantitation of flavonoids was done using HPLC-PDA, which is a more advanced method than the conventional methods of identification and quantitation using UV-VIS Spectrophotometer; it gives quantitation of total flavonoids rather than individuals (Glasl and Becker, 1984; Popova *et al.*, 2004).

LOD was three times of peak than background noise (S/N ratio: 3:1) and LOQ 10 times higher than the noise (S/N ratio: 10:1). LOD and LOQ were 0.0045 and 0.0135 $\mu\text{g/ml}$ and 0.25 and 0.75 $\mu\text{g/ml}$, respectively, for genistein and daidzein (Table 1). The results showed that, at a very low level, it is possible to identify and quantify the two isoflavonoids using LC-PDA. Linearity was satisfactory [r^2 : 0.999 and 0.997 for genistein and daidzein respectively], which was found from the calibration curves of the two certified reference samples.

A recovery experiment was done for determining the efficiency of the methods. The recovery of daidzein and genistein was 118 and 116% respectively, which are within the acceptable value according to the Codex Alimentary Commission (Uddin *et al.*, 2011).

Genistein and daidzein were identified by comparing

the retention times of the certified samples and the experimental samples, that is, soy-milk, masoor dal, and mung dal. Chromatograms of the certified samples and the study samples are shown in Figures 4 to 8.

All the analytical conditions of the certified isoflavones, in three different matrices, were kept same. The reference standard solutions were injected into the HPLC-PDA under the same condition of the cleaned extract of the food samples. Although the retention time of the standard and unknown samples was supposed to be same under the same analytical conditions, two peaks of the samples were same with the retention times of the standard compounds. Thus, this result clearly shows that we could identify the presence of isoflavones in the food samples.

Three replicate analyses were carried out for soy-milk, masoor dal, and mung dal. The mean and standard deviations of the samples were calculated, and the results are presented in Table 2.

The amount of daidzein and genistein in soy-milk was 36.25 $\mu\text{g}/100$ g of dry weight and 43.81 $\mu\text{g}/100$ g of dry weight respectively. The total amount of isoflavones was 80.05 $\mu\text{g}/100$ g of dry weight in soy-milk.

Although soybean has been cultivated in several places of Bangladesh, it is still not available in the market. India stands fifth in soybean production and sixth in terms of leading soybean-consuming countries in the world (Devi *et al.*, 2009). The total content of isoflavones in Indian soy-milk (Devi *et al.*, 2009) is much higher than that of

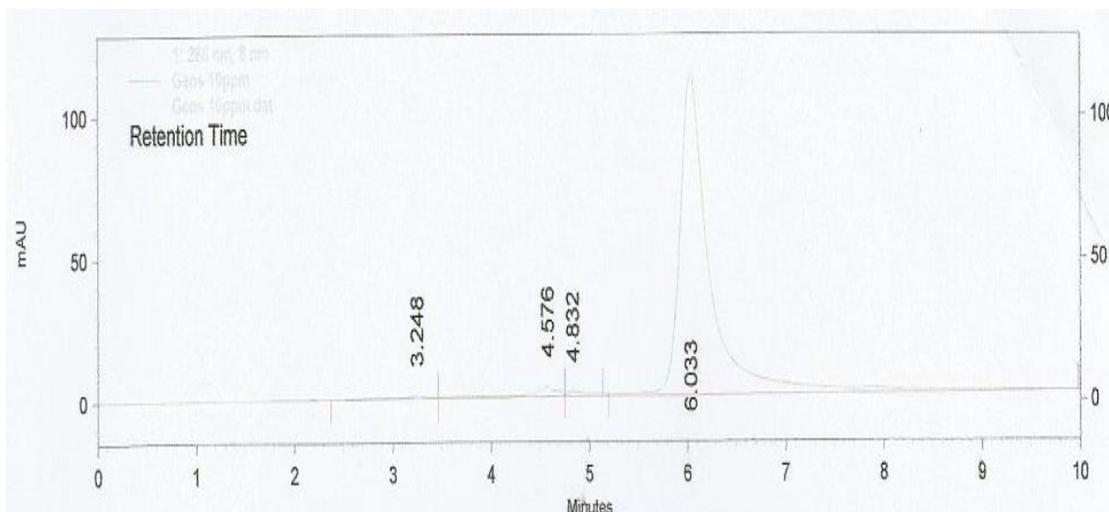


Figure 5. Chromatogram of genistein.

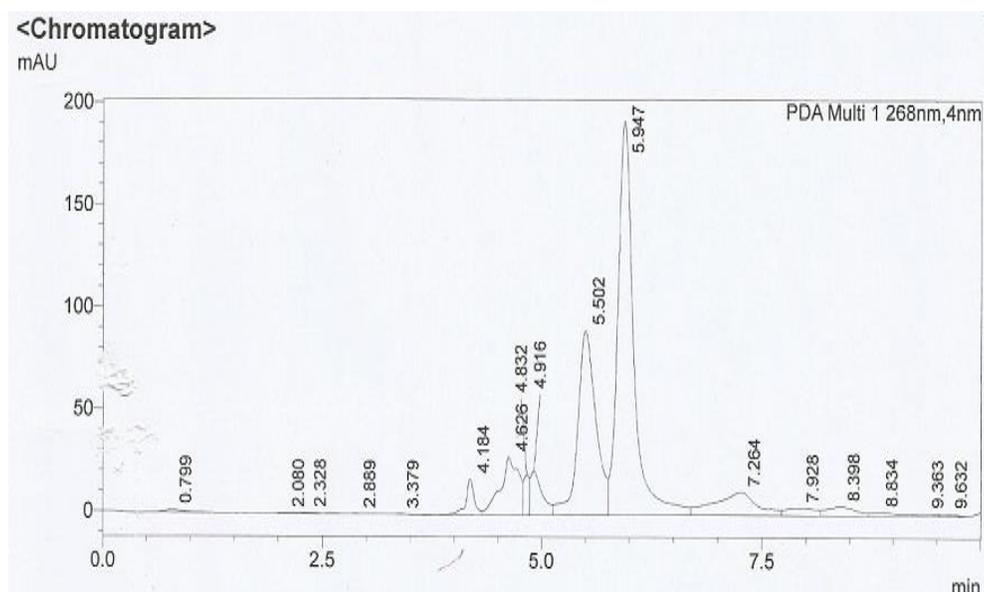


Figure 6. Chromatogram of soy-milk.

soybean produced in Bangladesh. In Singapore (Prabhakaran *et al.*, 2005), Korea (Kim and Kwon, 2001), Turkey (Orhan *et al.*, 2007; Gültekin, 2004), and the USA (Coward *et al.*, 1993), the total content of isoflavones in soy-milk is also higher than that of the study sample.

Dal, called the poor men's protein, is the most common foodstuff consumed by the people of Bangladesh. Masoor dal and mung (green gram) dal, though rich in isoflavones, are a non-soy food. The amounts of daidzein and genistein were 37.66 $\mu\text{g}/100\text{ g}$ of dry weight and 37.33 $\mu\text{g}/100\text{ g}$ of dry weight in masoor dal and 27.66 $\mu\text{g}/100\text{ g}$ of dry weight and 44 $\mu\text{g}/100\text{ g}$ of dry weight respectively in mung dal. The total amount of isoflavones was 74.99 $\mu\text{g}/100\text{ g}$ of dry weight in masoor dal and

71.66 $\mu\text{g}/100\text{ g}$ of dry weight in mung dal.

The total amount of isoflavones in mung dal produced in Bangladesh was found to be less than that of isoflavones in mung dal sample studied by (Mazur *et al.*, 1998). On the other hand, the total content of isoflavones in the masoor dal sample in our study was higher than that in masoor dal in the study of (Mazur *et al.*, 1998). Variations in contents and composition of isoflavones occur as a consequence of different factors among which the most examined ones are the genotype of the seed, the year, and the location of seeding (Wang and Murphy, 1994). The amount in cultivar's varieties is also dependent on the climatic conditions of the countries as well. The analysis of variance (ANOVA) test was performed to

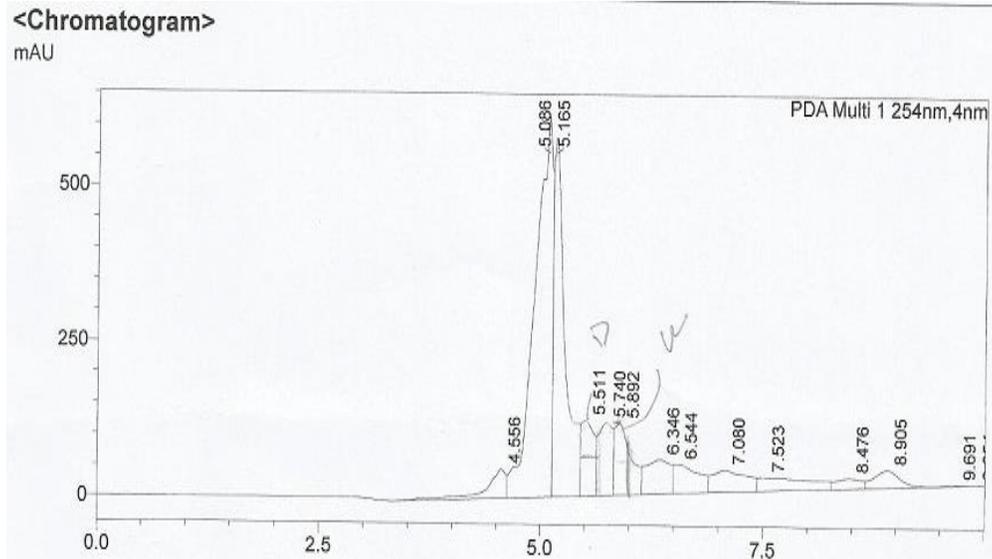


Figure 7. Chromatogram of masoor dal.

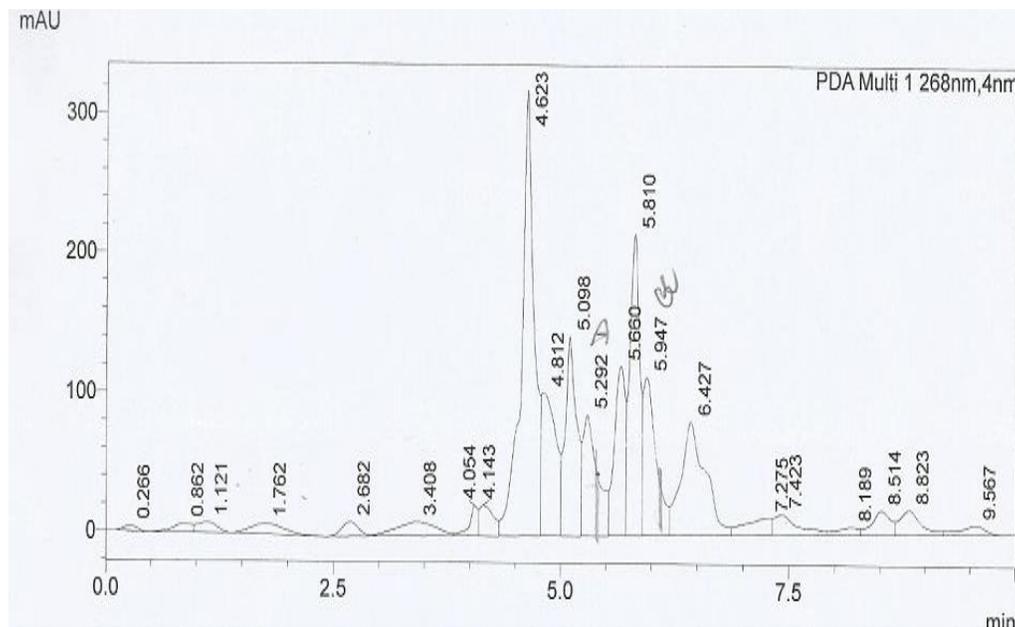


Figure 8. Chromatogram of mung dal.

compare the mean concentration of daidzein and genistein in the three food groups; p value ≤ 0.05 was considered significance, and no significant differences were observed in the mean concentration of daidzein and genistein in the three food items (soy-milk vs masoor dal vs mung dal). In the present study, the total amount of isoflavones in masoor dal and mung dal was found to be close to that of isoflavones (80.05 $\mu\text{g}/100$ g dry weight) in soy-milk.

The present study had, however, a couple of limitations. It was not possible to perform the extensive chemical analysis of isoflavones in the locally-produced

mung and masoor dals and in soybean due to the limited resources and financial and technical support.

CONCLUSION

It is apparent from the findings of the study that the presence of daidzein and genistein identified in the samples of locally produced soybean, masoor dal, and mung dal and the amount of total isoflavones in these samples are within the acceptable limit. It is, therefore, strongly recommended that different varieties of legumes,

Table 2. Amount of daidzein, genistein, and total isoflavones in soy-milk, *masoor dal*, and *mung dal*.

Food items	Daidzein ($\mu\text{g}/100\text{ g of powder}$)	Genistein ($\mu\text{g}/100\text{ g of powder}$)	Total isoflavones ($\mu\text{g}/100\text{ g of powder}$)
Soy-milk	36.25 \pm 2.67	43.81 \pm 4.37	80.05 \pm 6.87
Masoor dal	37.66 \pm 17.89	37.33 \pm 10.68	74.99 \pm 28.57
Mung dal	27.66 \pm 8.51	44.00 \pm 22.87	71.66 \pm 17.21
F/p	0.65/0.55	0.19/0.83	0.14/0.87
p-value			
Soy-milk vs. Masoor	ns	ns	ns
Soy-milk vs. Mung	ns	ns	ns
Masoor vs. Mung	ns	ns	ns

Results are expressed as mean \pm SD. One-way ANOVA (Post Hoc Bonferroni) is performed as the test of significance. * $p < 0.05$ is taken as level of significance; ns = not significant.

vegetables, and fruits produced in Bangladesh to be analyzed to know the content of isoflavones in them.

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Competing interests

The authors declare that they have no competing interests.

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