Changes of Phytoestrogens Daidzein, Genistein and Their Glycosides Daidzin and Genistin and Coumestrol during Processing of Soyabeans

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Abstract: Phytoestrogens represent biologically active compounds showing estrogenic activity similar to that of sex hormones – estrogens. Various adverse effects such as sterility, increase of females' genitals, lost of males' copulation activity, etc. were observed in farm animals after exposure to higher amounts of fodder containing phytoestrogens. On the other side, their presence in human diet is nowadays the object of many research studies concerned with prevention of breast and prostate cancer, osteoporosis and other hormone-linked diseases by dietary intake of phytoestrogens. Soya (*Glycine max*) is one of the main sources of these compounds in diet. Isoflavones daidzein and genistein occurring either free or bound in glycosides are the main phytoestrogens in this food crop. Coumesterol representing coumestans is another effective phytoestrogen contained in some edible plants. In the first part of our study, analytical method for determination of free and total phytoestrogens was developed and validated. Following steps are included: (i) acid hydrolysis (only for "total phytoestrogens" analysis), (ii) extraction with methanol/water mixture, (iii) SPE preconcentration; (iv) identification/quantification using HPLC/DAD/FLD. The aim of present study was to document the fate of phytoestrogens and their forms during household/industrial processing. As documented in our experiments the most dynamic changes of phytoestrogen levels occur during soyabeans sprouting. High levels of coumestrol even exceeding other phytoestrogens were detected on this occasion.

Keywords: soya; isoflavone; coumestrol; HPLC; household processing

INTRODUCTION

Isoflavones showing estrogenic activity occur in numerous plants used for human and animal nutrition. They are most abundant in soybeans, but are also present in considerable concentrations of beans, sprouts lowere levels occur also in legumes. Animal feed such as clover or alfalfa are significant sources of isoflavones. Figure 1 shows the structures of the most common dietary isoflavones. Their pattern varies among plants species; e.g. high levels of daidzein (DAI) and genistein (GEN) together with a small amount of glycitein are typical for soybeans, while red clover is rich in formononetin, biochanin A and coumestrol (CUM) (the later compound can be found in sprouting soya, too). In most plants, isoflavones are present as 7β-D-glycosides of glucose (daidzin (DIN) and genistin (GIN)) and 6″-*O*-malonylglucose. Upon ingestion by mammals, the aglycons are effective liberated from their glycoside forms and subjected, in part, to reductive metabolism by intestinal bacteria [1–3]. For example, typical bacterial metabolites of daidzein are dihydrodaidzein, *O*-desmethylangolensin and equol. The bacterial metabolites and the isoflavones not subjected to bacterial biotransformation are absorbed in the intestine, where extensive glucuronidation occurs in the enterocytes prior to release into the blood and transport to the liver [4]. Conjugates with glucuronic acid and sulphate are excreted into urine and bile. Biliary metabolites are known to undergo enterohepatic circulation [4].

The aim of this study was monitoring of selected phytoestrogens (daidzin, genistin, daidzein, genistein and coumestrol) during the most frequently

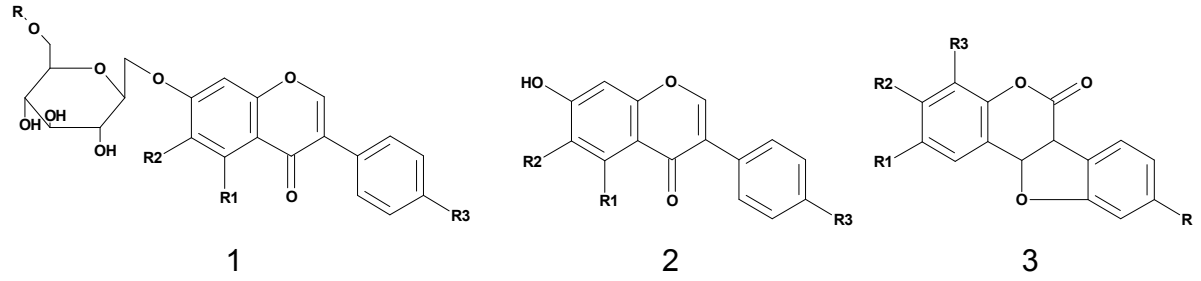

Figure 1. Structure of 1 – daidzin (R = H, R^1 = H, R^2 = H, R^3 = OH), genistin (R = H, R^1 = O, R^2 = H, R^3 = OH), 6′′-malonylgenistin (R = $COCH_2COOH$); 2 – daidzein (R^1 = H, R^2 = H, R^3 = OH), genistein (R^1 = OH, R^2 = H, R^3 = OH), formononetin (R^1 = H, R^2 = H, R^3 = OCH_3), glycitein (R^1 = H, R^2 = OCH_3, R^3 = OH), biochanin A (R^1 = OH, R^2 = H, R^3 = OCH_3); 3 – coumestrol (R = OH, R^1 = H, R^2 = OH, R^3 = H)

used culinary treatments to observe the influence of these factors on the content of phytoestrogens in processed beans.

Further common culinary treatments of soyabeans are soaking, cooking, roasting and sprouting.

EXPERIMENTAL

Culinary processing. (i) Soaking: soya beans were soaked for 4, 8, 12 hours in tap water 25°C. (ii) Cooking: soaked beans were cooked for 90 min in fresh water. (iii) Roasting: soaked beans were roasted 15 min in an electric oven at 200°C. (iv) Sprouting: soaked beans were placed on the surface of 10 mm wet layer cotton wool and held for 3 days at 25°C (cotton wool regularly changed daily).

Materials. Soybeans were purchased from retail market. Standards of isoflavones (DIN, GIN, DAI, GEN, CUM) were purchased from Sigma Aldrich Chemical Corp. (USA). HPLC-grade methanol from Merck (Germany) were used. All other chemicals were purchased from LACHEMA Ltd. (Czech Republic). The working standard solutions were prepared by dilution in HPLC mobile phase and stored in fridge at 4°C. All samples were filtered through a 0.5 μm Teflon membrane filters (MetaChem, Torrance, CA, USA) before HPLC separation.

Extraction methods, glycosides and free aglycones. Homogenized sample – 1 g (aliquot taken from 250–500 g) was three times sonicated in 20 ml of 80% (v/v) methanol for 30 min. Combined extracts after filtration were transfered into 50 ml volumetric flask. 1 ml of methanolic extract after dilution with 3 ml of deionized water was used for SPE extraction.

Acid hydrolysis, total daidzein and genistein. Homogenized sample (1 g) was mixed with 10 ml of hydrochloric acid (6 mol/l) and 40 ml of 98% (v/v) ethanol. The mixture was heated for 4 h at 65°C under reflux. 1 ml of extract was used for SPE clean up.

SPE clean-up. SPE procedure was performed using automatic SPE system ASPEC XL (Gilson, France). Oasis HLB cartridge (Waters, USA) was conditioned with 5 ml of methanol and 5 ml of water, coextracts were washed out with 5 ml of 10% methanol (v/v) and target analytes were eluted with 8 ml of methanol. The extract was evaporated to dryness. The residues were dissolved in 1 ml of mobile phase.

Identification and quantification. HPLC analysis were performed using Hewlett-Packard HP 1100 (Hewlett-Packard, USA) liquid chromatograph equipped with DAD and FLD detectors. Separation was carried out on LiCrospher CN column (250 × 4 mm, 5 μm particles) with LiCrospher CN precolumn (4 × 4 mm, 5 μm particles). The mobile phase was methanol:buffer (citric acid – potassium citrate, pH 3). The linear gradient was follows 0 min 30% methanol (v/v); 30 min 65% methanol (v/v). The flow rate was 1 ml/min. Daidzin, genistin, daidzein, genistein were detected at 260 nm and coumestrol at 343 nm with DAD. Coumestrol was detected also with FLD detector (Ex 245 nm and Em 420 nm). The limit of quantification was 0.4–0.6 mg/kg for DIN, GIN, DAI and GEN. The limit of quantification for coumestrol was 0.02 mg/kg (extract was concentrated 20 times). Repeatability (RSD) of the method was in the range 4–8% for all target analytes.

Table 1. Levels of phytoestrogens (in mg/kg) in raw samples of soya beans before processing*

Statistics.	Daidzin	Genistin	Daidzein		Genistein	
			free	total	free	total
Mean	338.1	351.4	4.4	710.6	5.4	744.7
Range	248.8	236.9	2.6	229.8	1.1	213.5
Standard deviation	83.3	79.6	0.9	151.4	0.5	122.1
Confidence interval	67.9	64.9	0.7	123.4	0.4	99.5

*content of coumestrol was less than LOD (0.02 mg/kg)

RESULTS AND DISCUSSION

The content of target analytes in raw soya beans was measured in 8 parallel samples (4 × 250 g homogenized sample; 2 parallels of each). Mean, standard deviation and confidence interval were calculated on the basis of these results. Further, two parallel measurements of samples after processing were carried out. The levels of target analytes in processed samples were compared with levels in raw samples. The target analytes contents in raw soya beans are shown in Table 1.

From obtained statistical datas it is evident, that the content of measured phytoestrogens in material is variable. It is necessary to consider this fact during interpretation of obtained results. The results of all of the analytes were adjusted to dry mass.

Soaking

While no significant changes in total daidzein/genistein and their glycosides dadzin/genistin content were observed during soaking (Table 2), free daidzein and genistein content increased significantly during this process (daidzein 14×, genistein 8×). This can be due to their release from glucosides and/or ester glucosides, at the same time the decrease of genistin and dadzin can be compensated by their formation from ester glucosides as the result of cleavage of the ester bond with malonic acid (Table 2).

Cooking

30% (GEN) and 50% (DAI) reduction of total daidzein and genistein content was observed in cooked soya beans, however the amount of respective glucosides slightly increased, similarly, higher levels of free aglycones (2.5–3 times as compared to original soaked beans) were determined in processed samples. Alike in previous case, several processes are obviously taking part simultaneously; the resulting levels depend on the extent of leaching of target compounds into boiling water as well as on breakdown of glycosidic and ester

Table 2. Changes of daidzein, genistein and their glycosides daidzin and genistin and coumestrol (in mg/kg) during processing of soyabeans – dry mass*

Processing	Daidzin	Genistein	Daidzein		Genistein		Coumestrol
			free	total	free	total	
No processing	375.6 ± 67.9	390.4 ± 64.9	4.9 ± 0.7	789.5 ± 123.4	6.0 ± 0.4	827.4 ± 99.5	LOD
Soaking	369.5 ± 58.9	421.5 ± 56.3	69.4 ± 0.6	739.4 ± 107.1	47.7 ± 0.3	828.2 ± 86.3	LOD
Cooking	468.2 ± 58.9	618.1 ± 56.3	13.8 ± 0.6	362.0 ± 107.1	16.0 ± 0.3	546.7 ± 86.3	LOD
Roasting	534.9 ± 58.9	476.2 ± 56.3	102.6 ± 0.6	837.2 ± 107.1	78.0 ± 0.3	845.8 ± 86.3	LOD
Sprouting 1 day	249.6 ± 58.9	280.0 ± 56.3	37.3 ± 0.6	643.4 ± 107.1	26.1 ± 0.3	760.2 ± 86.3	0.5 ± 0.5
Sprouting 2 days	320.2 ± 58.9	418.2 ± 56.3	54.0 ± 0.6	728.4 ± 107.1	47.7 ± 0.3	857.1 ± 86.3	3.1 ± 1.9
Sprouting 3 days	325.5 ± 58.9	335.9 ± 56.3	81.6 ± 0.6	862.6 ± 107.1	47.3 ± 0.3	872.5 ± 86.3	7.7 ± 2.7

*confidence interval for parallel measurements after processing is interpreted as SD/$\sqrt{2}$, where *SD* is standard deviation for raw beans; LOD 0.02 mg/kg

bonds in compounds containing bound daidzein and genistein (Table 2).

Roasting

High termostability of daidzein and genistein was demonstrated in this experiment. The total daidzein and genistein content did not change during roasting, however extensive increase of free aglycons occurred (daidzein up to 21×, genistein 13×) the changes of glycosides were not pronounced (Table 2).

Sprouting

As regards phytoestrogens, the most dramatic changes observed during sprouting of soaked soybeans was exponencial increase of coumestrol: 30× during the first day, the content in the third day was even 400× (bean + sprout), the highest levels were in the surface layer of been. Compared to coumestrol the increase of free genistein and daidzein was not so extensive: 7–16 times (daidzein) and 4–8 times (genistein). The levels of measured glycosides and the total content of daidzein and genistein varied only slightly during sprouting (Table 2).

CONCLUSION

The above experiments documented large changes in the daidzein and genistein forms (bound in glycosides and/or ester glycosides vs free) during procedures employed for household/industrial processing of soybeans. The only procedure resulting in the decrease of total phytoestrogens content was boiling. Generaly relative content of free glycosides increased. Dramatic increase of coumestrol was observed during sprouting. Due to its high estrogenic potential this fact should be considered when assessing risks/benefits associated with soy-based products dietary intake.

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