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Free Isoflavone (Daidzein and Genistein) Content in Soybeans, Soybean Meal and Dried Soy Hypocotyl Sprout using High Performance Liquid Chromatography (HPLC)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Phytoestrogens are natural plant glucosides found primarily in legumes and grains. Phytoestrogens can interact with oestrogen receptors in the body either by mimicking or blocking the effects of oestrogen. Isoflavones are a major subclass of phytoestrogens and often comprise a

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substantial part of livestock feeds. This study was focused on the detection and quantification of free isoflavones especially genistein and daidzein in soy-based ingredients (soybeans, soybean meal and dried soy hypocotyl sprout) which are incorporated in the animal feed formulations.

Methods and Results: The simultaneous determination of daidzein and genistein in soybeans, soybean meal and dried soy hypocotyl sprout was carried out using the analytical technique high performance liquid chromatography (HPLC). The limits of detection and quantitation were 4.81 and 14.59 µg/100 g for daidzein and 3.06 and 9.58 µg/100 g for genistein respectively. The precision of the procedure was 2.73 per cent for daidzein and 2.88 per cent for genistein. The average daidzein and genistein concentration levels were (239.16 ± 0.21 and 238.68 ± 0.27 µg/100g) for soybeans, (397.24 ± 0.64 µg/100g and 443.85 ± 0.62) for soybean meal, (551.03 ± 0.54 and 742.59 ± 0.76 µg/100g) for dried soy hypocotyl sprout. The mean recovery percentages for daidzein and genistein were 92.8 per cent and 93.66 per cent respectively. The findings indicated that all the soy-derived components examined contained isoflavones and dried soy hypocotyl sprouts possessed higher concentrations of both daidzein and genistein.

Keywords: Daidzein; genistein; soybean; isoflavones; phytoestrogens.

1. INTRODUCTION

Phytoestrogens are nonsteroidal heterogenous group of herbal substances with a spatial conformation similar to oestradiol 17β [1]. The chemical structure of phytoestrogens makes it possible for their affinity, selectivity and efficacy to bind with oestrogen receptors. Hence isoflavones can also be used as an alternative to external oestrogen substitutes or in hormone replacement therapy.

Genistein and daidzein are the predominant isoflavones and together make more than 90% of the total isoflavones in soy-based food [2]. In plants, they occur in general as glycosides, which are deconjugated by intestinal β glucosidases to release the aglycones [3].

The health benefits of the isoflavones daidzein and genistein and concerns about potential adverse effects have also been raised. Numerous epidemiological and experimental studies in animals and humans had shown that phytoestrogens can be oestrogenic or antiestrogenic, antioxidative, antiproliferative, antiviral, antibacterial, insecticidal or fungistatic, cardioprotective, antiatherogenic, hypocholesterolemic, bone-maintaining and anticarcinogenic [2].

Soybean products are a good source of isoflavones and consumed worldwide as both food and food additives in processed foods to enhance texture, flavour and nutritional content [4]. Soybean meal is the main source of plant-derived protein as well as animal protein substitute which is incorporated in animal/poultry feeds up to 25 per cent by weight.

This research investigates the potential for the transfer and accumulation of these soy-derived isoflavones from the feed into milk, eggs and other edible tissues of livestock species.

Currently, HPLC equipped with Diode array detector (DAD) and reverse-phase C18 stationary matrices is the most sensitive and reliable analytical technique used for the determination of daidzein and genistein in soy-based ingredients of animal feed formulations.

2. MATERIALS AND METHODS

2.1 Experimental Layout

The detection and quantification of free isoflavones in soy based ingredients by HPLC was conducted in the department of Veterinary Physiology and Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

Six samples of soybeans and soybean meal used for the analysis were procured randomly from local markets who supply animal feed ingredients for various feed mills. Soybean meal is a high protein product after oil extraction, produced at industrial or laboratory scale. Soybean meal extracts were obtained either by ethanol or a ternary solvent mixture composed of water, ethanol and ethyl acetate (40:40:20).

Sprouting is a common procedure to enhance the nutritional value of seeds. Soy hypocotyl sprout samples were harvested on the fifth day of germination by sowing soybean seeds in the soil after soaking them for at least 8 hours and then subjected to drying and subsequent analysis.

2.2 Determination of Free Isoflavone (Genistein and Daidzein) Content

2.2.1 Standards for HPLC

Chemicals and solvents of The High-Performance Liquid Chromatography (HPLC) grade were procured from M/s Thermo Fisher Scientific, Massachusetts. Genistein and daidzein standards were obtained from M/s Sigma Aldrich, US.

Stock solutions of free isoflavones (genistein and daidzein) were prepared in volumetric flasks by dissolving 2 mg of standard in 2 mL of HPLC grade methanol (99.9%). For the quantification and recovery tests, composite working standard solutions of 1 to 500 µg/mL were prepared by diluting the stock solution with suitable quantities of methanol [5].

Two different gradient elution system were used as mobile phases to separate genistein and daidzein from samples [6]. The gradients included an aqueous solvent A (0.1% acetic acid, 5% acetonitrile in water) and an organic solvent B (0.1% acetic acid in acetonitrile).

After preparation, the mobile phase container was placed in an ultra sonicator for nine minutes to ensure thorough mixing and then filtered through a solvent filtration unit under vacuum using a membrane filter (0.45µm pore size - M/s Pall Corporation, New York).

2.2.2 Sample preparation to estimate genistein and daidzein content

The method reported by Lin et al. [7] was modified to quantify isoflavones in the collected soy-based samples. One gram of sample was weighed and extracted with 10 mL of 80% methanol. The samples were placed on an incubator orbital shaker for 1 h. The mixture was centrifuged in high speed refrigerated microcentrifuge for 20 min and supernatants were collected. The precipitates were re-dissolved in 80% methanol and placed on the orbital shaker for an additional 30 min. Samples were re-centrifuged and collected supernatants were pooled. The extracted volume was evaporated to < 2mL in an incubator at 40ºC. Extracts were mixed with 10mL of sodium acetate (0.1*M*, pH 5) and 75µL of β-glucuronidase (*Helix pomatia*, 1000 Fishman units). The solution was incubated overnight at 37 ºC and finally passed through C-18 purification kit.

2.2.3 Clean up technique

All the samples were finally purified by passing them through C- 18 cartridges [8] using a C-18 extraction and purification kit (Bond Elut 500mg/3mL). Samples were purified by washing the column first with 10% acetonitrile in water (4 mL) and with 0.1 % acetic acid (2 mL) to remove the more polar compounds. Isoflavones were recovered from the cartridge with 80% methanol and followed by a mixture of ethyl acetate and methanol in a ratio of 80:20 (2 mL).

2.2.4 Final sample preparation

After C-18 clean up procedure, the samples were concentrated by evaporation and finally, extracts were reconstituted with 20% methanol to a volume of 2mL, then filtered through a 0.45µm syringe polypropylene filter and injected (20 µL) into the HPLC system. Each sample was run in triplicate.

2.2.5 Chromatographic conditions

The genistein and daidzein concentrations in the samples were detected and quantified by HPLC and Chromeleon software interfaced to the computer.

Standard chromatogram was generated for each injected standard and test sample using DAD of chromatograph with a time window of 28 minutes for genistein and daidzein The column temperature was held constant at 35˚C. Twenty microliters of blank (mobile phase), standards and test samples were injected separately into the C18 column (3µm, 2.1x 150 mm) using an autosampler. The samples were separated by reverse phase column elution using the mobile phase at a flow rate of 1 mL/min. Following the injection of 20 µL sample, a gradient program was implemented for the mobile phases of both genistein and daidzein (Table 1), as recommended by Saitoh et al. [9]. The UV-VIS detection was carried out at 262 nm wavelength which was specific for genistein and daidzein.

2.3 Development and Validation of an Hplc Method for Analysis

2.3.1 Linearity of genistein and daidzein concentration versus area curve

The squared correlation coefficient is defined as linearity, which was calculated from the standard curve using Microsoft Excel. Ascending standards of daidzein and genistein @ 1 to 500

Table 1. Gradient elution programme for daidzein and genistein detection

Time (min)	Flow rate	Percent Mobile phase B
0	1mL	0
1	1mL	15
5	1mL	27.5
25	1mL	32.5
28	1mL	0

µg/mL in methanol were analysed using HPLC for the construction of calibration plots. From the chromatograph, peak area was calculated for each dilution, and concentration was plotted against the peak area. The reproducibility of the result was verified at least thrice with each concentration of standards. The regression was done using MS-Excel® and regression equation was found.

2.3.2 Precision of the method by intra-day variation of spiked concentrations

Precision of the method employed was calculated by intra-day variation in percentage recovery of daidzein and genistein standards from samples. For intraday variation, the samples were spiked with 1000 µg/mL of standard before the extraction procedure and recovery was estimated. The experiment was repeated thrice a day and precision was expressed by coefficient of variation.

2.3.3 The limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection is one of the measurements of sensitivity, defined as the quantity yielding a detector response which gives signal to noise ratio 3:1, according to the International Conference on Harmonisation (ICH). The limit of detection is the lowest concentration in a sample that can be detected, but not quantified, from background noise.

Limit of quantification is one of the measurements of sensitivity, defined as the lowest amount that can be analysed within acceptable precision and accuracy which gives signal to noise ratio 10:1 according to ICH. The limit of quantification is the lowest concentration of analyte with a signal to noise ratio of at least 10 and can be determined with acceptable accuracy and precision.

2.4 Statical Analysis

The data obtained was analysed by the one way ANOVA using software SPSS version 24.0.

3. RESULTS AND DISCUSSION

The genistein and daidzein isoflavones in soybeans, soybean meal and dried soy hypocotyl sprout were separated in HPLC by reverse phase affinity gradient elution using solvent A (0.1% acetic acid, 5% acetonitrile in water) and an organic solvent B (0.1% acetic acid in acetonitrile) as mobile phase.

3.1 Calibration Curve

Standard solutions were injected three times into the HPLC column in triplicate and the mean peak area was calculated using chromeleon software version 6.80. Chromatogram of different concentrations of daidzein and genistein standards are shown in Fig. 1. The retention time (RT) of standards was found to be in and around 12.24 to 12.28 minutes for daidzein and 18.99 to 19.14 minutes for genistein. The sample peak fraction coinciding with the above mentioned retention time were corresponding to that of daidzein and genistein.

The height and area of the peaks were found to be increased with increasing concentration of standards. For estimation of daidzein and genistein calibration curve with the standards 1.95, 3.90, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL were constructed as shown in Fig. 2 and Fig. 3 respectively. The calculated values of genistein and daidzein standards were back referred to the expected values and were depicted in the Table 2.

3.2 Linearity of Isoflavone Standard Concentrations Versus Area Curve

Ascending concentrations of daidzein and genistein: 1.95, 3.90, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL were analysed using HPLC and the peaks with area were calculated. Linear regression analysis was done using MS-Excel®. A good linearity of the method was observed with a correlation coefficient of 0.999 between the concentration range of 1 µg/ mL to 500 µg/ mL for daidzein and genistein. The

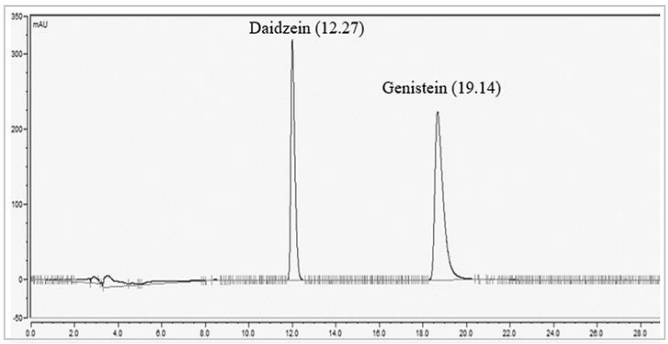

Fig. 1. Chromatogram of eluted daidzein and genistein standard 500 ug/mL

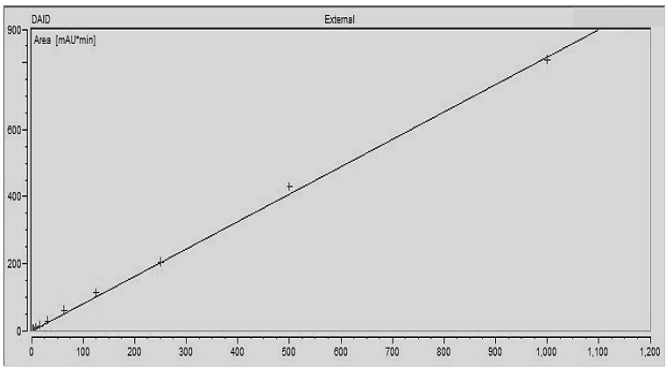

Fig. 2. Calibration curve prepared by plotting peak area (Y axis) versus daidzein concentrations (µg/mL) (X axis) over a range of 1 to 500 µg/mL

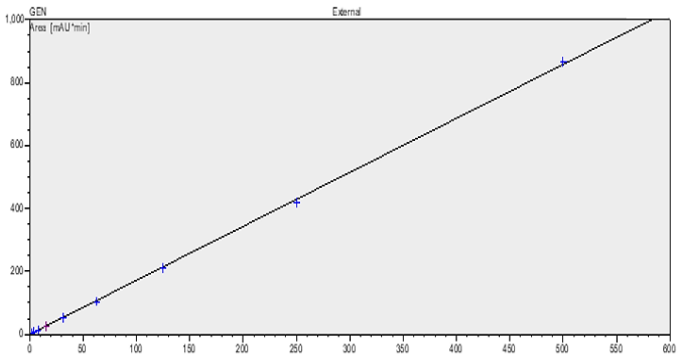

Fig. 3. Calibration curve prepared by plotting peak area (Y axis) versus genistein concentrations (µg/mL) (X axis) over a range of 1 to 500 µg/mL

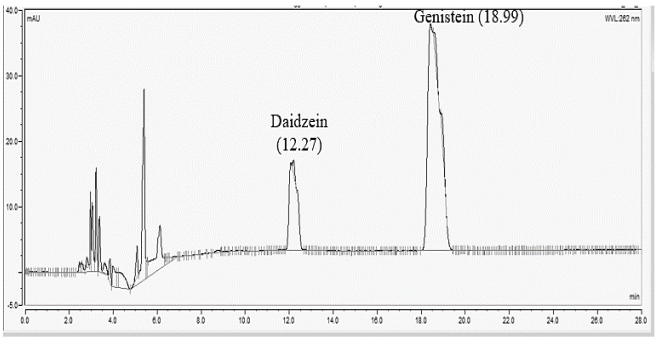

Fig. 4. Representative chromatogram of isoflavones in dried soy hypocotyl sprout

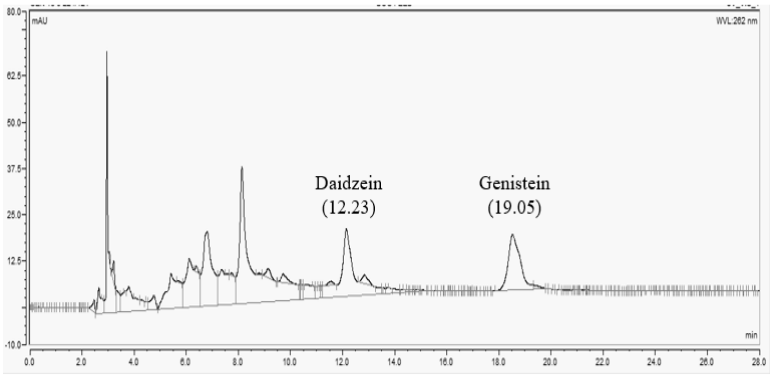

Fig. 5. Representative chromatogram of isoflavones in soy bean meal

Table 2. HPLC retention time, peak area, expected concentration and calculated amount of daidzein and genistein standards

Daidzein				Genistein			
Concentration	**RT**	**Area**	**Calculated amount**	**Concentration**	**RT**	**Area**	**Calculated amount**
1.953125	12.28	1.86	2.272	1.953125	19.00	3.37	1.961
3.90625	12.27	3.84	4.689	3.90625	19.00	6.62	3.858
7.8125	12.28	7.47	9.126	7.8125	18.99	12.91	7.521
15.625	12.28	13.90	16.980	15.625	18.99	24.77	14.431
31.25	12.28	28.09	34.316	31.25	19.00	51.06	29.751
62.5	12.24	58.44	71.383	62.5	19.00	101.98	59.418
125	12.27	112.14	136.979	125	19.00	209.71	122.185
250	12.27	203.47	248.550	250	19.02	418.05	243.572
500	12.27	428.93	523.966	500	19.14	865.79	504.439

Table 3. Recovery of 1000 µg/mL of isoflavone standards

Spiked concentration of Phytoestrogens	**1000 µg/mL (Daidzein)**	**1000 µg/mL (Genistein)**
Recovery % at 8.00 a.m.	92.00	90.80
Recovery % at 12.00 p.m.	96.25	94.00
Recovery % at 4.00 p.m.	90.20	96.18
Mean Recovery %	92.8 ± 1.46	93.66 ± 1.56
Standard deviation	2.54	2.70
Co-efficient of Variation %	2.73	2.88

unknown concentration of free isoflavones (genistein and daidzein) were quantified using the regression equations, 0.8123x + 4.2551 for daidzein and 1.7257x - 3.1151 for genistein.

3.3 Precision of Method by Intraday Variation of Spiked Concentrations

The intra-day variation was carried out for 1000 µg/mL concentration of daidzein and genistein standards in three replicates at different time intervals in the same day. The precision of procedure was 2.73 % and 2.88 % for genistein and daidzein respectively as given in Table 3.

3.4 Limit of Detection and Limit of Quantification

It was observed that the LOD of this method was 4.81, 3.06 µg/100g for daidzein and genistein respectively with acceptable accuracy and precision. The method demonstrated a limit of quantification (LOQ) of 14.59 µg/100g for daidzein and 9.58 µg/100g for genistein, which is determined consistent with guidelines.

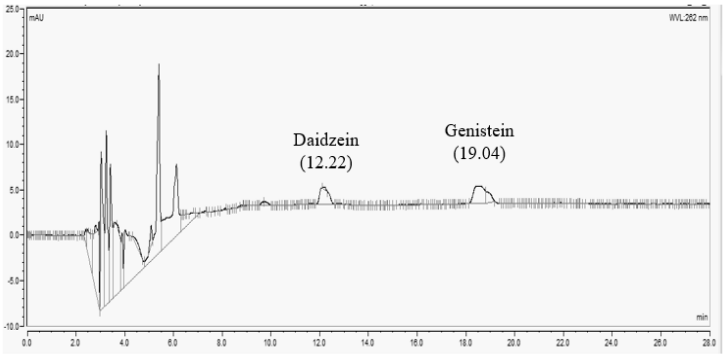

Fig. 6. Representative chromatogram of isoflavones in soy beans

Table 4. Mean ±S.E. values of daidzein and genistein concentration in samples

Treatment group	Mean value (µg/100g) in samples	
	Daidzein	Genistein
Soy bean seed	239.16[a] ± 0.21	238.68[a] ± 0.27
Soy bean meal	397.24[b] ± 0.64	443.85[b] ± 0.62
Dried soy hypocotyl sprout	551.03[c] ± 0.54	742.59[c] ± 0.76
p-Value	0.00*	0.03*

Means with different superscripts in the same column differ significantly
** Significant (p <0.05)*

3.5 Identification of Free Isoflavone (Genistein and Daidzein) Content in Feed Samples

The Table 4 presents free isoflavone (genistein and daidzein) content of soy-based samples. Genistein and daidzein content were significantly different ($P < 0.05$) for soybeans soybean meal and dried soy hypocotyl sprout. Higher mean values of daidzein and genistein were detected in soybean sprout (Fig. 4) followed by soybean meal samples (Table 4). On the other hand, soybean seed samples showed the lower mean value for both daidzein and genistein compared to soybean meal and dried soy hypocotyl sprout.

Soybean is mentioned to be one of the most important cultivated legumes throughout Asia. Soy supplements are the richest source of isoflavones with concentrations ranging from 118 to 306 mg/100g and these were the common ingredients of laboratory animal feed [10].

Similar to the present findings, Wang and Murphy [11] recorded isoflavone concentration of 1.2 to 3.3 mg /g dry weight in unprocessed soybeans. On contrary Eldridge and Kwolek [12] found a higher concentration of total isoflavone in soybeans which ranged from 116 to 309 mg/g within the variety and varied from 46 to 195 mg/g in the same variety grown at different locations.

According to the study by Liggins et al. [13], soy-based products contained considerable amounts of isoflavones. Among these products, soybeans had the highest concentration, ranging from 14 to 153 mg/100 g. Soy foods and some foods with soybean additives had the largest concentration of these oestrogenic compounds and were marketed because of their cost effectiveness [14].

Grgic et al. [15] noticed that soybean had an average isoflavone concentration of 0.7 to 5.2 g/ kg dry weight. The maximum concentration was 102 mg/kg for genistein and 465 mg/kg for its glucosidic conjugate. The corresponding values for daidzein and its glucosidic conjugate were similar but lower compared to genistein and genistin.

Moreover, high moisture thermal processing of soybeans being a procedure that is employed to improve the functional value of the product as it seemed to yield meals with potentially higher isoflavones bioavailability. The current study findings were also supported by de Oliveira Silva and Perrone [16] who reported a higher concentration of daidzein (0.09 mg/g dry weight)

and genistein (0.08 mg/g dry weight) in soybean meals compared to soybeans.

In the research conducted by Sudar et al. [17], the total isoflavone content in soybean meal was ranged from 67.27 to 98.39 mg/100g depending on the applied extraction conditions of pressure and temperature. The most abundant isoflavone was genistein. The predominance of genistein in our research study is consistent with the same observation.

Soybean meal provided approximately 150 µg daidzein/g and 250 µg genisten/g as per the reports of Dixon and Ferreira [18]. Flachowsky et al. [19] determined the isoflavone concentration of defatted soybean meals from Argentina, Brazil and USA. The average total isoflavone concentrations in mg/ kg dry matter in soybean meal were 1570 from Brazil, 1944 from the USA and 3075 from Argentina.

Sprouting is one of the processing methods to enhance bioavailability of nutrients of agricultural products. According to the reports of Gilani and Anderson [20], soy products prepared from the hypocotyledons were found to have >20 mg/g of isoflavones. The trend of higher isoflavone concentration in dried soy hypocotyl sprout in this research was in agreement with the studies of Tsukamoto et al. [21] who observed that compared to cotyledons, hypocotyls displayed higher concentrations of isoflavones on dry weight basis.

The isoflavone content of the cotyledons changed significantly in response to high temperatures, while the content in the hypocotyls remained high during seed development. However, total isoflavone content and its subtypes was found to be depended on plant variety, geographical location, year of harvest, maturity and environmental conditions [22].

4. CONCLUSION

The objective of this study was to determine the isoflavone concentration in soybeans, soybean meals and dried soy hypocotyl sprout which are commonly employed in animal feed production. Free isoflavones (genistein and daidzein) concentration was significantly higher in dried hypocotyl sprout followed by soybean meal and seeds.

This study has shown that soy-based products, particularly dried hypocotyl sprouts, provide an additional source of isoflavones in the diet of animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Woclawek-Potocka I, Mannelli C, Boruszewska D, Kowalczyk-Zieba I, Wasniewski T, Skarzynski DJ. Diverse effects of phytoestrogens on the reproductive performance: Cow as a model. International Journal of Endocrinology. 2013;1–15. Available:https://doi.org/10.1155/2013/650984
2. Nilsson S, Gustafsson JA. Biological role of estrogen and estrogen receptors. Crit. Rev. Biochem. Mol. Biol. 2002;37:1-28.
3. Kuhnle GGC, Dell'Aquila C, Aspinall SM, Runswick SA, Mulligan AA, Bingham SA. Phytoestrogen content of foods of animal origin: Dairy products, eggs, meat, fish, and seafood. Journal of Agricultural and Food Chemistry. 2008; 56(21): 10099–10104. Available:https://doi.org/10.1021/jf801344x
4. Cassidy A. Committee on Toxicity draft report on phyto-oestrogens and health–review of proposed health effects of phyto-oestrogen exposure and recommendations for future research. Nutrition Bulletin. 2003;28(2):205-213. Available:https://doi.org/10.1046/j.1467-3010.2003.00346.x
5. Hu L, Jin K, Zheng B, Yang X, Lei S. Simultaneous Determination of isoflavones and equol in egg yolk using UPLC-MS/MS. Food Analytical Methods. 2019;12: 859–868. Available:https://doi.org/10.1007/s12161-018-01417-z
6. Saitoh S, Sato T, Harada H, Matsuda T. Biotransformation of soy isoflavone-glycosides in laying hens: Intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic

metabolite of daidzein. Biochimica et Biophysica Acta (BBA)-General subjects. 2004;1674(2):122-130.
Available:https://doi.org/10.1016/j.bbagen.2004.06.006

7. Lin F, Wu J, Abdelnabi MA, Ottinger MA, Giusti MM. Effects of dose and glycosylation on the transfer of genistein into the eggs of the Japanese quail (*Coturnix japonica*). Journal of Agricultural and Food Chemistry. 2004; 52(8): 2397–2403.
Available:https://doi.org/10.1021/jf034921f
8. Pettersson H, Kiessling KH. Liquid chromatographic determination of the plant estrogens coumestrol and isoflavones in animal feed. Journal of the Association of Official Analytical Chemists. 1984;67(3): 503-506.
Available:https://doi.org/10.1093/jaoac/67.3.503
9. Saitoh S, Sato T, Harada H, Takita T. Transfer of soy isoflavone into the egg yolk of chickens. Bioscience, Biotechnology and Biochemistry. 2001;65(10):2220-2225.
Available:https://doi.org/10.1271/bbb.65.2220
10. Boettger-Tong H, Murthy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Makela S. A case of a laboratory animal feed with high estrogenic activity and its impact on *In vivo* responses to exogenously administered estrogens. Environmental Health Perspectives. 1998; 106(7):369-373.
Available:https://doi.org/10.1289/ehp.98106369
11. Wang HJ, Murphy PA. Isoflavone content in commercial soybean foods. Journal of Agricultural and Food Chemistry. 1994;42(8):1666-1673.
Available:https://doi.org/10.1021/jf00044a016
12. Eldridge AC, Kwolek WF. Soybean isoflavones: Effect of environment and variety on composition. Journal of Agricultural and Food Chemistry. 1983; 31(2):394-396.
Available:https://doi.org/10.1021/jf00116a052
13. Liggins J, Mulligan A, Runswick S, Bingham SA. Daidzein and genistein content of cereals. European Journal of Clinical Nutrition. 2002;56(10):961-966.
Available:https://doi.org/10.1038/sj.ejcn.1601419
14. Vargas Galdos DMM. Quantification of soy isoflavones in commercial eggs and their transfer from poultry feed into eggs and tissues. Doctoral thesis, The Ohio State University. 2009;61.
Available:http://rave.ohiolink.edu/etdc/view?acc_num=osu1236706764
15. Grgic D, Varga E, Novak B, Muller A, Marko D. Isoflavones in animals: Metabolism and effects in livestock and occurrence in feed. Toxins. 2021;13(12): 836(1)-836(33).
Available:https://doi.org/10.3390/toxins13120836
16. De Oliveira Silva F, Perrone D. Characterization and stability of bioactive compounds from soybean meal. LWT-Food Science and Technology. 2015; 63(2):992-1000.
Available:https://doi.org/10.1016/j.lwt.2015.04.032
17. Sudar R, Jokic S, Velic D, BiliC M, Sudaric A, Kelekovic S, Zrinusic SZ. Determination of isoflavones in soybean meal after supercritical carbon dioxide extraction. Proceedings of 47th Croatian and 7th International Symposium on Agriculture, Opatija, Croatia. 2012;561- 564.
18. Dixon RA, Ferreira D. Genistein. Phytochemistry. 2002; 60(3):205-211.
Available:https://doi.org/10.1016/S0031-9422(02)00116-4
19. Flachowsky G, Hunerberg M, Meyer U, Kammerer DR, Carle R, Goerke M, Eklund M. Isoflavone concentration of soybean meal from various origins and transfer of isoflavones into milk of dairy cows. Journal fur Verbraucherschutz und Lebensmittelsicherheit. 2011;6:449-456.
Available:https://doi.org/10.1007/s00003-011-0702-7
20. Gilani GS, Anderson JJB. (Eds.), Phytoestrogens and health. The American Oil Chemists Society Press, Champaign, Illinois, USA; 2002.
21. Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K. Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. Journal of Agricultural and Food Chemistry. 1995;43(5):1184-1192.

Available:https://doi.org/10.1021/jf00053a012

22. Stevenson L. Effects of the soy phytoestrogen genistein on the reproductive development of immature female broiler chickens. Doctoral dissertation, Auburn University. 2007;135. Available:http://hdl.handle.net/10415/825

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