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The level of isoflavones (genistein and daidzein) in different chicken feed formulations

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Abstract
The objective of the present study was to detect and quantify the level of isoflavones (genistein and daidzein) in different chicken feeds which were formulated based on BIS standards 2007. Six samples of chick mash, chicken grower and chicken breeder feeds used for analysis were procured from poultry feed mills and the samples were incubated with β-glucuronidase for better detection of different isoflavone aglycones and metabolites. The extracted isoflavones were purified by C18 solid phase extraction and analysed by high performance liquid chromatography (HPLC). The mobile phase composition, gradient elution programme and injected volume were optimised for separation efficiency and sensitivity. It is an accurate and precise analytical methodology which shows good reproducibility and can be easily adapted to detect isoflavones.
The average genistein concentrations were 279.85 ± 1.30, 84.48±1.97 and 148.26 ± 1.64 µg/100 g in chick mash, chicken grower and chicken breeder respectively. However, the average daidzein concentration was in 276.29 ± 1.57 µg/100 g chick mash, 51.67±1.11 g/100 g in chick grower and 153.16 ± 1.21 µg/100 g in chick breeder. The findings indicated that the concentration of genistein and daidzein in the feeds was positively correlated with the level of soybean in the feed since it is the predominant source of isoflavones.

Keywords: Genistein, daidzein, chick mash, chicken grower, chicken breeder, soybean meal

Introduction
Isoflavones are potent phytoestrogens which have been widely studied because of their health benefits in modulating endocrine functions. Soybean meal constitutes a main ingredient of livestock feed formulations and soy isoflavones content have been evaluated in animal products due to their transfer from the diet. Isoflavones reside as glycosides with low estrogenic activity compared to their deglycosylated aglycone form. Upon ingestion, these compounds are metabolically hydrolysed by the intestinal microflora to their aglycones thus potentially mediating an estrogenic stimulus.
Chick mash, chicken grower and chicken breeder feeds are types of specialized poultry feeds designed to meet the nutritional needs of chickens at different stages of their life cycle. These feeds are formulated to provide the appropriate balance of nutrients for chicks, growing chickens and breeding chickens to support their growth, health and reproduction.

Materials and Methods
Experimental diet
Chick mash is a type of starter feed designed for newly hatched chicks up to approximately 8 weeks of age. It is typically in the form of finely ground pellets or crumbles to make it easy for young chicks to consume. Chicken grower feed is intended for chickens that have outgrown the chick stage but are not yet ready for breeding or laying eggs. It is suitable for chickens from 9 to 16 weeks of age. Chicken breeder feeds are formulated specifically for mature chickens that are used for breeding purposes, whether for meat production or egg laying. The average incorporation level of soybean meal (SBM) was 30, 13 and 20 percent respectively in chick mash, chicken grower and chicken breeder feeds. The feed formulation of chick mash (CM), chicken grower (CG) and chicken breeder (CB) are enlisted in table 1.

Table 1: Formulation of chick mash (CM), chicken grower (CG) and chicken breeder (CB) feeds

Ingredients	CM	CG	CB
Maize	48.2	52.4	53
Dorb	10	22.7	15.5
SBM	29.9	13.1	20.3
Cacite	2.2	2.2	4
Grit	0	0	5
Dcp	1.5	1.6	1.3
Methionine	0.2	0.2	0.2
Lysine	0.1	0.4	0.2
Soda Bicarb	0	0	0.15
Salt	0.5	0.5	0.4
Wheat Bran	7.5	7	
Total	100	100	100
Feed Additives			
Vitamin Premix	50	50	75
Toxin Binder	100	100	100
Livertonic	30	30	25
Natchol	50	100	200
Tmmix	125	10	150
Avizyme	35	35	35
Coccistac	50	50	0

Standards and mobile phase for HPLC

All the chemicals and reagents of analytical grade were procured from M/s Sigma Aldrich India Pvt. Ltd. and M/s Merck Specialities Pvt. Ltd., Banglore. Stock solutions of free isoflavones (genistein and daidzein) were prepared in volumetric flasks by dissolving 2 mg of standard in 2 mL of HPLC- MS grade methanol (99.9%). Quantification and recovery tests were carried out with composite working standard solutions of 1 to 500 µg/mL which were prepared by diluting the stock solution with suitable quantities of methanol (Hu *et al*., 2019) [6]. Two different gradient elution systems were used as mobile phases to separate genistein and daidzein from samples (Saitoh *et al*., 2004) [8]. 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).

Feed sample preparation to estimate genistein and daidzein content

One gram of feed was weighed and extracted with 10 mL of 80 percent methanol. The samples were placed on an incubator orbital shaker for 1 h. The mixture was centrifuged twice @ 4500 rpm in high speed refrigerated microcentrifuge for 20 min. Extracts were mixed with 10mL of sodium acetate and 75 µL of β-glucuronidase. The solution was incubated overnight at 37 °C and finally passed through C-18 purification kit before analysis.

Chromatographic and mass spectrometric conditions

Standard chromatogram and mass spectra were generated for each injected standard and test sample using DAD of chromatograph with a time window of 28 min. The column temperature was held constant at 35 °C. Twenty microliters of blank (mobile phase), samples prepared from control birds, standards and test samples were injected separately into the C18 column (3µm, 2.1X 150 mm) using an autosampler. The genistein and daidzein were separated by reverse phase column elution using a gradient program @ flow rate of 1 mL/min. The UV-VIS detection was carried out at 262 nm and 260 nm wavelength which was specific for genistein and daidzein.

Results

Chromatogram of genistein and daidzein standards are shown in figure 1. The height and area of the peaks were found to be increased with increasing concentration of standards. 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 for estimation of isoflavones. The retention time (RT) of standards was found to be in and around 18.99 to 19.14 min. for genistein, 12.24 to 12.28 min. for daidzein.

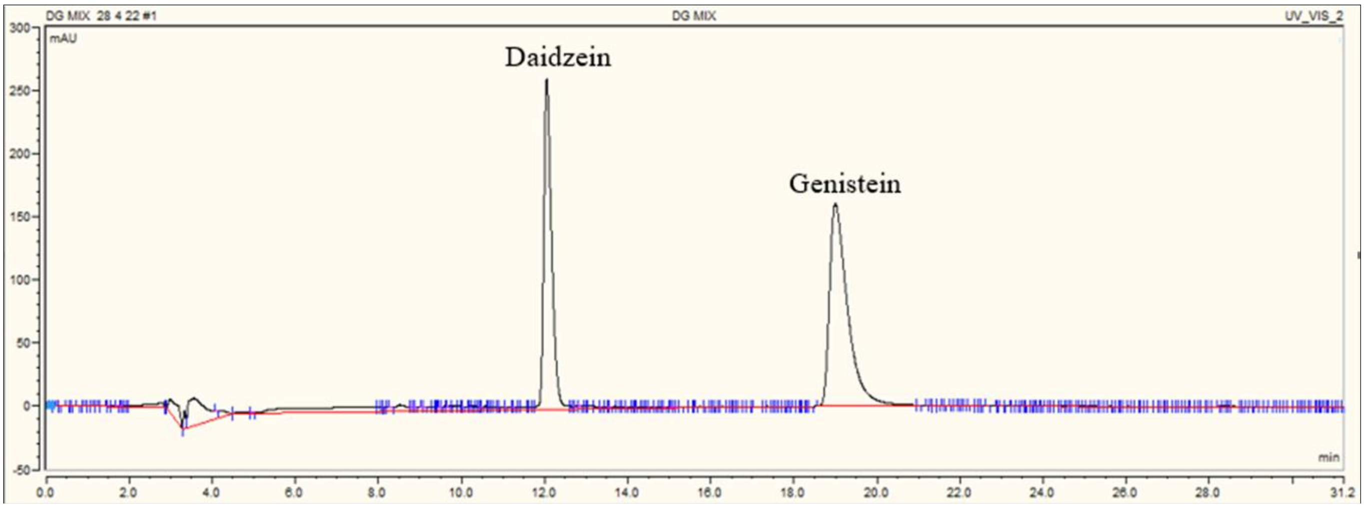

Fig 1: Chromatogram of daidzein and genistein standards

Genistein and daidzein content of feed samples were significantly different ($p \leq 0.05$) among the groups. Higher mean values of genistein and daidzein were detected in chick mash (Fig. 2) feed samples followed by chicken breeder (Fig. 3) feed samples. On the other hand, chicken grower (Fig. 4) feed samples showed lower mean value for both genistein and daidzein. The table below (Table 2) presents the findings regarding the impact of soybean meal supplementation on the isoflavone content of feed samples.

Table 2: Mean ± S. E. value of isoflavones (genistein and daidzein) content in feed samples

Groups	Mean value (µg/100 g) in feed samples	
	Genistein	Daidzein
Chick Mash	279.85[a] ± 1.30	276.29[a] ± 1.57
Chicken Grower	84.48[c] ± 1.97	51.67[c] ± 1.11
Chicken Breeder	148.26[b] ± 1.64	153.16[b] ± 1.21
p-Value	≤ 0.05	≤ 0.05

Means with different superscripts in the same column differ significantly

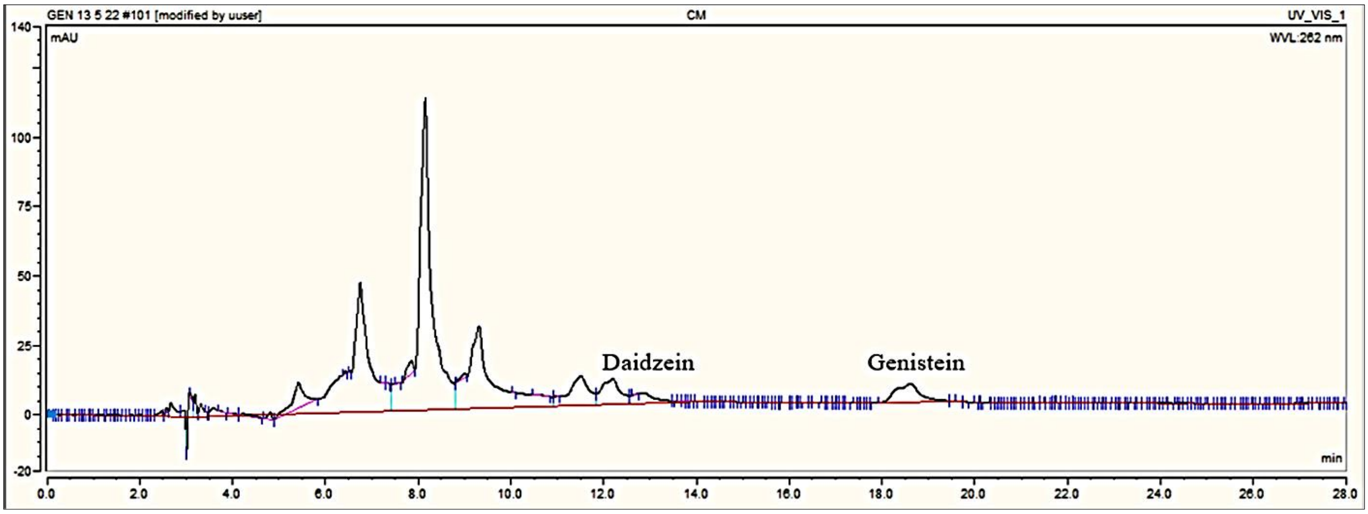

Fig 2: Representative chromatogram of genistein and daidzein in chick mash feed

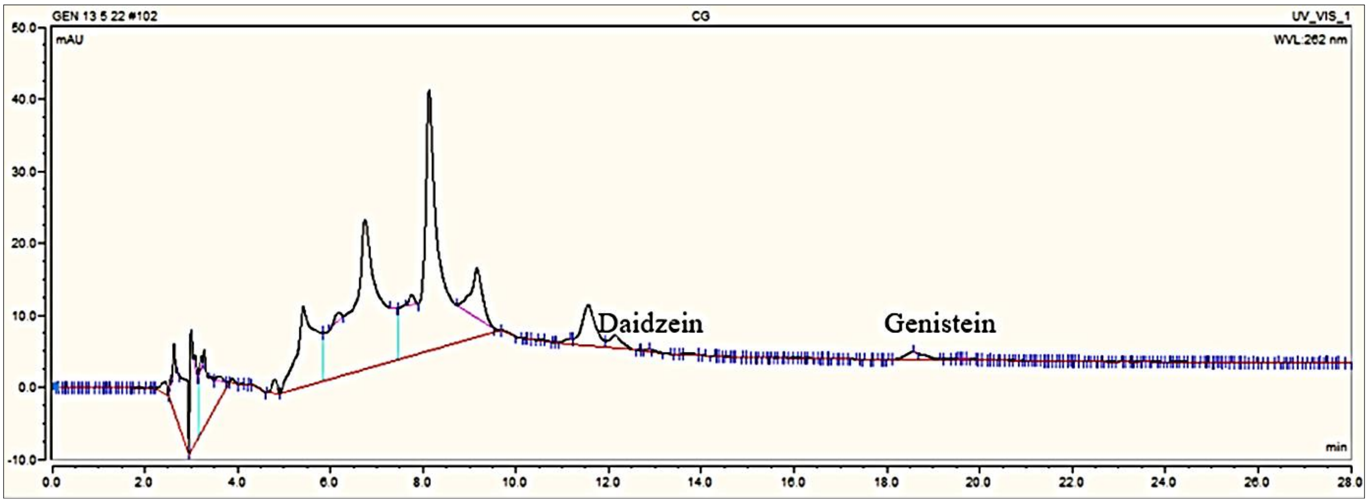

Fig 3: Representative chromatogram of genistein and daidzein in chicken grower feed

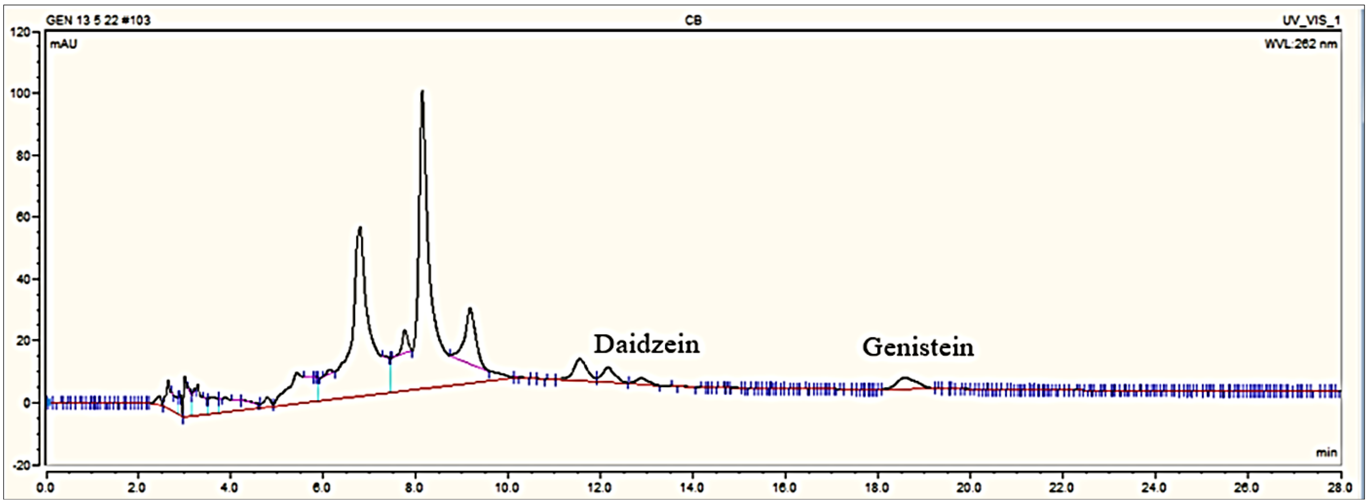

Fig 4: Representative chromatogram of genistein and daidzein in chicken breeder feed

A non-significant positive correlation was observed between the SBM level and genistein content of feeds. However, it was significant and positively correlated between SBM and daidzein (Table 3).

Table 3: Correlation between SBM and isoflavone content of CM, CG and CB

SBM / Isoflavone	SBM	
	Correlation coefficient	p-Value
Genistein	0.981	0.126
Daidzein	0.998	0.035

Discussions

Soybean meal is a source of unsaturated fatty acids for energy utilization in livestock, especially in the poultry feed industry. Soy products are commonly used as animal feed due to their high protein content of about 38%, healthful fat and high fibre. This was the major driving force that stimulated the production and consumption of soybeans all over the world.

The accuracy of identifying and quantifying isoflavones in feed samples were greatly influenced by the specific extraction and purification procedures and conditions (Gilani and Anderson, 2002) [4]. The most prevalent dietary isoflavones include genistein, daidzein, glycitein, formononetin, and biochanin A. These compounds are primarily found in legumes where they occur as glucosides (Liggins *et al*., 2002) [7]. Generally, soybean meal provided approximately 150 µg daidzein per gram and 250 µg genistein per gram (Dixon and Ferreira, 2002) [3].

As per the review of Grgic *et al*. (2021) [5], isoflavones were detected in 43% of cattle feed samples collected from January 2019 to April 2021 (n = 542) and the prevalence of the individual isoflavone in cattle feed ranged from 2.6% to 31%. Isoflavones were also detected in 45% of the pig feed samples, with daidzin and genistin as well as their aglycones being the most prevalent ones. The detected maximum concentrations were 140.0 mg/kg for genistein.

Grgic *et al*. (2021) [5] also detected the presence of soy isoflavones in 88% of the poultry feed samples (n=263). Aglycones were detected in lower concentrations compared to the respective glycosides. The maximum concentrations were 102 mg/kg for genistein and 465 mg/kg for its glucoside genistin. The corresponding values for daidzein and its glucoside daidzin were similar but somewhat lower compared to genistein and genistin. Formononetin and biochanin A showed only low prevalence in poultry feed (0.8 and 5%, respectively).

Ciftci (2017) [11] noticed that genistin and daidzin remained stable even when subjected to high temperatures during soybean processing and they were not lost during the traditional soybean processing methods. As a result, when domesticated poultry were fed with diets containing corn and soy led to complete absorption of all the genistin and daidzin present in soybeans which might be a contributing factor for higher isoflavone content in the different chicken feeds.

The current study findings were also supported by Silva and Perrone (2015) [9] who reported that high moisture thermal processing of soybeans being a procedure that was employed to improve the functional value of the product as it assumed to yield meals with potentially higher isoflavones bioavailability.

Soybean is mentioned to be one of the most important cultivated legumes throughout Asia Soy supplements are the richest source of isoflavones (Boettger-Tong *et al*., 1998) [1] with concentrations ranging from 118 mg/100 g to 306 mg/100 g. Therefore, soy foods and some foods with soybean additives had the largest concentration of these estrogenic compounds and were marketed because of their cost effectiveness (Vargas Galdos, 2009) [10].

Conclusions

The free isoflavones such as genistein and daidzein were identified in different chicken feed formulations. The significantly higher mean values of genistein and daidzein were detected in chick mash feed followed by chicken breeder and grower feeds and it was positively correlated with the level of soybean meal incorporated in the feed.

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