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High Performance Liquid Chromatography (HPLC)-Based Detection and Quantification of Free Isoflavones (Daidzein and Genistein) in Livestock Feed Ingredients

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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

Phytoestrogens are naturally occurring plant compounds, predominantly found in legumes and grains. They can bind to estrogen receptors in the body, either imitating or inhibiting the effects of oestrogen. This study aimed to detect and quantify free isoflavones, specifically genistein and daidzein, which are found in commonly used feed ingredients such as maize, soybean meal (SBM), de-oiled rice bran (DORB), groundnut cake (GNC), gingelly oil cake (GOC), dried fish and wheat bran, which are often included in livestock and poultry feed formulations.

Feed ingredients commonly used in livestock feed mills were randomly selected from the market and analysed for daidzein and genistein levels using high-performance liquid chromatography (HPLC) at the Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur. The average concentrations of daidzein varied from undetectable (UD) to 567.94 µg/100g, while genistein levels ranged from UD to 551.02 µg/100g across the various feed ingredients. The findings of this study revealed that soybean meal contained significantly higher levels of both daidzein and genistein compared to other ingredients used in livestock and poultry feed formulations. This study provides crucial data on the levels of phytoestrogens in common livestock feed ingredients, which can impact animal health and productivity. It is one of the first to comprehensively quantify isoflavones like genistein and daidzein across a wide range of feed ingredients, particularly in the context of animal nutrition. The findings can be used to optimise feed formulations, ensuring that phytoestrogen levels are managed to support the desired outcomes in livestock and poultry production.

Keywords: High performance liquid chromatography; isoflavones; phytoestrogen; soybean meal.

1. INTRODUCTION

Phytoestrogens represent the group of natural products that, due to the structural similarity with the female sex hormones, have the biological activity as estrogens [1]. Soybean is the main source of plant derived phytoestrogen especially in dairy cows, pigs, goats and poultry species and it substitutes animal proteins like bone meal. The health benefits of isoflavones have been described and should make it desirable to increase soybean/isoflavone consumption [2]. The major driving force that has stimulated the production and consumption of soybean all over the world is the use of soybean meal as high-quality source of protein and unsaturated fatty acids for utilising energy by the animal in livestock feed industry [3,4].

Dietary isoflavones found in soybeans are mainly the glycosidic forms of three base aglycones (daidzein, genistein and glycitein). After consumption isoflavones are initially hydrolyzed and absorbed in the small intestine into the liver where most are glucuronidated (70%). Isoflavone absorption depends greatly on the individuals' microflora. Generally, soybean meal provides approximately 150 µg daidzein per gram and 250 µg genistein per gram [5]. However, phytoestrogens contained in soybean can differ greatly from crop to crop, region to region [6].

It is possible that isoflavones could be transferred and/or accumulated into animal products, which could become additional sources of dietary isoflavones for humans [7]. Diets rich in plant-derived products may supply a variety of phytoestrogens capable of producing various pharmacological effects in the human body. As humans live longer, women particularly are spending more of their lives in menopause, affected by a variety of estrogen-related conditions such as osteoporosis, cognitive and cardiovascular decline, increased risk of breast cancer and other symptoms that decrease the overall quality of life [8]. It has been widely noted that individuals from countries with high dietary content of soy foods are less likely to develop mammalian cancer [9].

Soy isoflavone supplementation during late-gestation and lactation improves reproductive performance and serum antioxidant status of sows, and enhances the growth performance of their offspring in a dose-dependent manner [10]. Isoflavone-enriched feed in dairy cows leads to reduced microbial richness and fewer bacteria in the rumen compared to control cows [11]. Soy-derived bioactive compounds show potential as immunomodulatory feed additives for pigs, improving their immune status and growth performance under certain disease challenges [12]. Feeding extruded full-fat soybeans to lactating dairy cows significantly increases the concentration of phytoestrogens daidzein and genistein in milk and plasma, resulting in higher milk yield and composition [13]. Adding 2.5% soybean oil to wheat bran-based diets improves laying hen performance by reducing feed conversion per egg mass [14].

Screening for isoflavones in feed ingredients is essential to ensure that their levels are controlled, optimising the health and productivity of livestock while also considering the potential transfer of these compounds to humans through animal products. High-performance liquid chromatography (HPLC) is advantageous for detecting isoflavones because of its precision, sensitivity, and ability to accurately separate and quantify these compounds compared to traditional methods.

2. MATERIALS AND METHODS

Table 1 provides the typical percentages of various feed ingredients commonly incorporated into livestock and poultry feed formulations. Feed ingredients were obtained from various feed mills associated with Kerala Veterinary and Animal Sciences University, India representing typical sources for livestock and poultry feed formulations in the region. The ingredients were randomly selected from the local markets within the same geographic area to ensure consistency. Each type of feed ingredient was collected in quantities of approximately 1 kilograms to ensure sufficient material for thorough analysis. The samples were transported to the Central Instruments Laboratory at the College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala in sealed, sterile containers to prevent contamination and degradation before analysis.

2.1 Feed Sample Preparation to Estimate Genistein and Daidzein Content [15]

One gram of feed was extracted with 10 mL of 80% methanol and shaken on an orbital shaker for 1 hour. The mixture was centrifuged at 4500 rpm for 20 minutes, and the supernatant was collected. The extract was evaporated to under 2 mL at 40 ºC, then mixed with 10 mL of sodium acetate (0.1M, pH 5) and 75 µL of β-glucuronidase. After overnight incubation at 37 ºC, the solution was purified using a C-18 column, and the feed samples were analyzed for free isoflavones using HPLC. These feed ingredients are processed in the Central Instruments Laboratory at the College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India.

2.2 Chromatographic and Mass Spectrometric Conditions

Stock solutions of free isoflavones (genistein and daidzein) were prepared by dissolving 1 mg of each isoflavone standard in 1 mL of HPLC-MS grade methanol (99.9%) in volumetric flasks. Quantification and recovery tests were conducted using composite working standard solutions ranging from 1 to 500 µg/mL, diluted from the stock solution with methanol. Gradient elution systems (Fig. 1) were used as mobile phases: an aqueous solvent A (0.1% acetic acid, 5% acetonitrile in water) and an organic solvent B (0.1% acetic acid in acetonitrile) to separate genistein and daidzein. Chromatograms were generated for each standard and sample using Diode Array Detector (DAD) detection over a 30-minute window. Separation was achieved via reverse-phase column elution with a mobile phase flow rate of 1 mL/min and Ultraviolet-Visible spectroscopy (UV-VIS) detection was performed at 262 nm. This procedure was previously described by [16] and has been adapted and modified.

Table 1. Proportions of different feed ingredients in livestock feed formulations

No	Feed ingredients	Per Cent of Incorporation															
		CM	**CG**	**CB**	**DS**	**DG**	**DB**	**CS**	**DC**	**PC**	**PG**	**PF**	**LA**	**RA**	**HS**	**GT**	**DG**
1	Yellow maize	48	52	53				35	14	38	48	36	10	20	20	12	4.5
2	De-oiled rice bran	10	23	16	19	20	9		24	15	15	5		18	16	27	
3	Soybean meal	30	13	20	20	10	20	10		18	10	20	17		5		16
4	Groundnut cake																
5	Wheat bran	7.5	7			7		20		18	15	24	10				9
6	Gingelly oil cake							8	17			5	20	6		25	4.6
7	Dried fish meal				2	2	5					8		17	13		

CM: Chick mash
CG: Chicken grower
CB: Chicken breeder
DS: Duck strater
DG: Duck grower
DB: Duck breeder
CS: Calf starter
DC: Dairy cattle
PC: Pig creep
PG: Pig grower
PF: Pig finisher
LA: Lab Animal
RA: Rabbit
HS: Horse
GT: Goat
DG: Dog

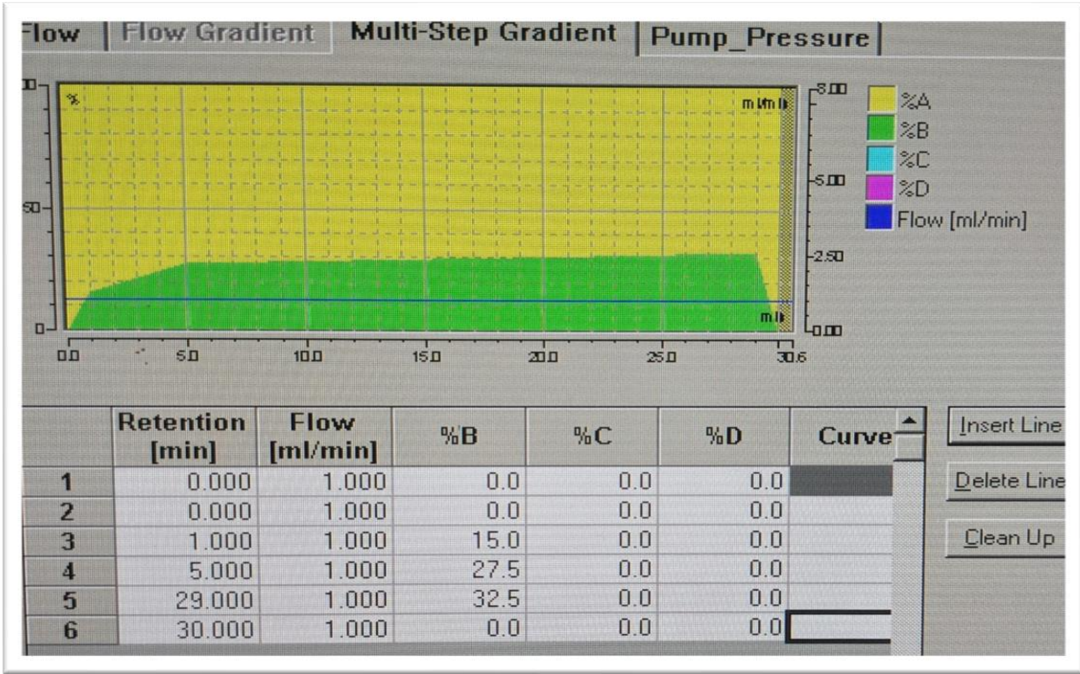

Fig. 1. Gradient elution programme for genistein and daidzein

3. RESULTS AND DISCUSSION

This study likely represents a pioneering effort in estimating the levels of phytoestrogens in livestock feed ingredients, an area that has not been extensively researched. Due to the limited research available in this field, this work could provide valuable insights and contribute significantly to the understanding of phytoestrogens' effects in animal nutrition

Phytoestrogens are present in all animal-based foods, providing a more accurate estimation of dietary exposure to these compounds [17]. Phytoestrogen content is a valuable biomarker for distinguishing plant-derived raw materials used in fish feed production, with rapeseed meal, soybean meal, sunflower meal, and wheat meal showing the highest correlations with their origin descriptions [18]. Phytoestrogens in legumes can affect reproductive functions in grazing livestock, but proactive management strategies can help mitigate potential fertility loss and improve growth and performance [19].

In this study, both peak height and area increased proportionally with the concentration of the standards. Fig. 2 illustrates the chromatogram of genistein and daidzein standards. Calibration curves were generated using standard concentrations of 1.95, 3.90, 7.81, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL to quantify isoflavone levels (Fig. 3 and Fig. 4). Isoflavones were studied as possible growth promoters for use in the animal feed industry in the 1950s due to their estrogenic effects in rodents [20]. The research study of Hua [21] developed a rapid HPLC method for detecting soybean isoflavone components in feeds, providing guidance for livestock and poultry production.

The chromatogram illustrating the detection of varying levels of daidzein and genistein in different livestock feed ingredients, with each compound corresponding to its specific retention time (RT) are represented in Fig. 5. The chromatographic analysis consistently revealed that the retention times for genistein and daidzein standards fell within the precise ranges of 18.99 to 19.14 minutes and 12.24 to 12.28 minutes, respectively. These retention times were used as reference points to identify and quantify the presence of these isoflavones in the feed samples analyzed. The consistent retention times indicate the reliability and precision of the chromatographic method used in the study for the detection and quantification of these specific isoflavones in various livestock feed ingredients.

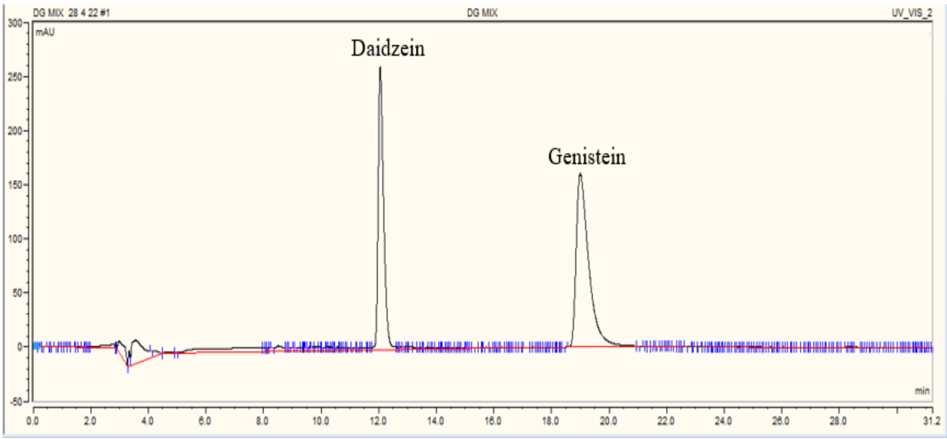

Fig. 2. Chromatogram of daidzein and genistein standards
[15]

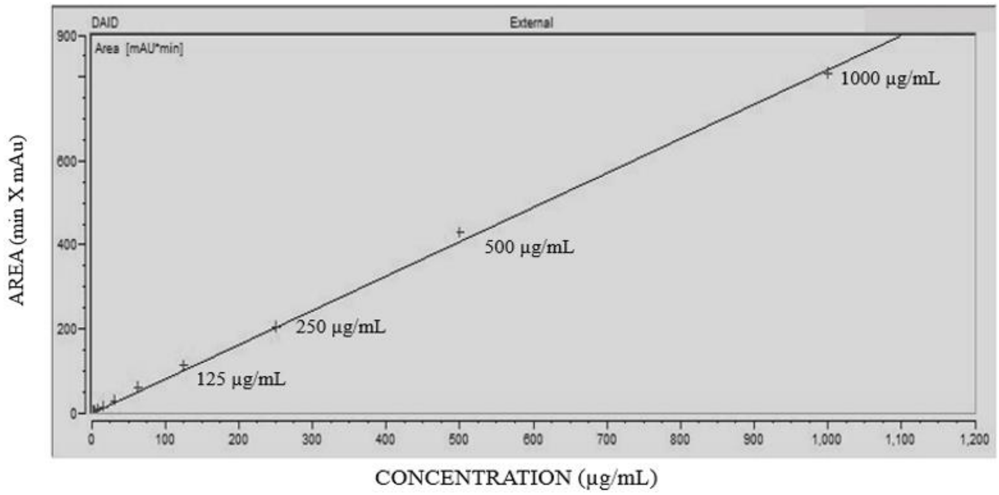

Fig. 3. Calibration curve of daidzein with different standard concentrations

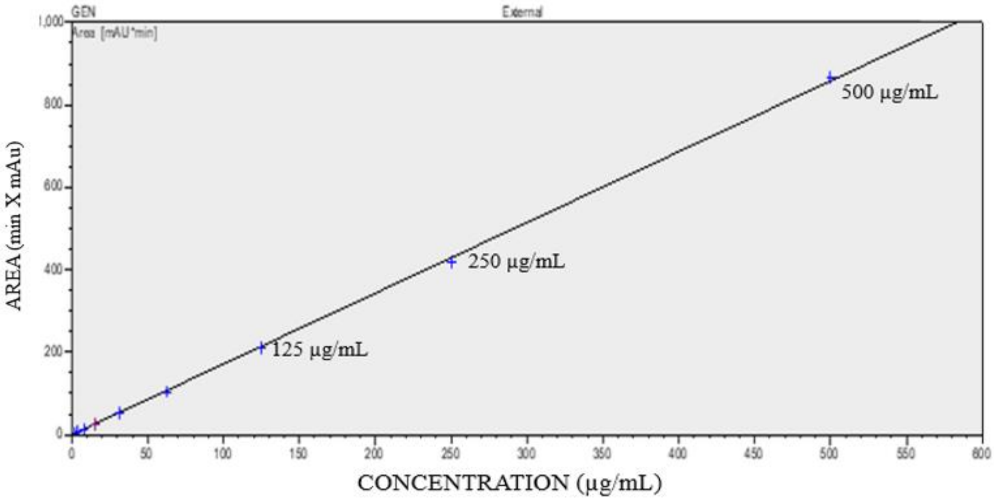

Fig. 4. Calibration curve of genistein with different standard concentrations

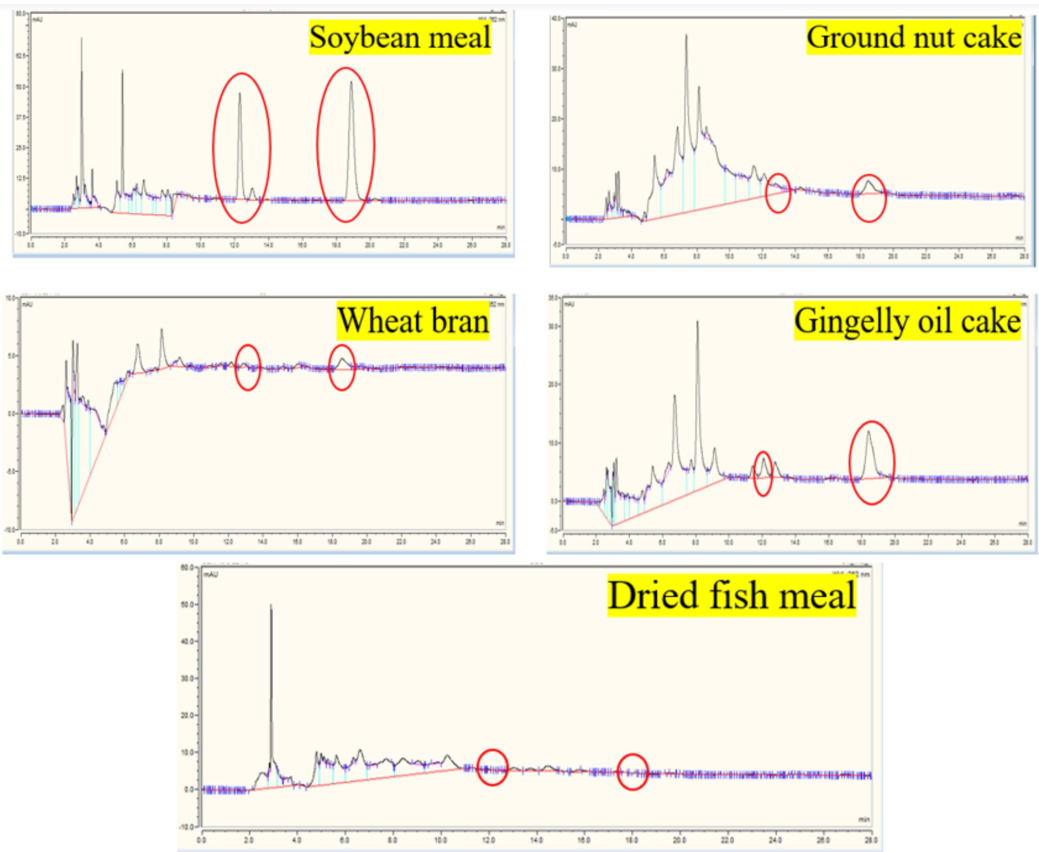

Fig. 5. Isoflavone detection in feed ingredients: Sample chromatograms

Table 2. Isoflavone levels in livestock feed ingredients

Ingredients	Concentration (µg/100g)	
	Daidzein	**Genistein**
Yellow maize	0.76	0
Deoiled rice bran	127.60	198.03
Soybean meal	567.94	551.03
Ground nut cake	0.39	198.61
Dried Fish Meal	0	0
Wheat bran	0.513	183.66
Gingelly oil cake	30.98	186.44

The quantification of free isoflavones, specifically daidzein and genistein, in various livestock feed ingredients are mentioned in Table 2. High concentrations of both daidzein (567.94 µg/100g) and genistein (551.03 µg/100g) were detected in soybean meal, making it a significant source of isoflavones in livestock feed. Soybean seed isoflavone levels exhibit a location-specific response, with temporal variability between years being more influential than location [22]. Other ingredients such as deoiled rice bran, groundnut cake, wheat bran, and gingelly oil cake showed moderate levels of genistein and varying levels of daidzein. Yellow maize contained a minimal amount of daidzein with no detectable genistein, while dried fish meal had no detectable levels of either isoflavone. This data highlights the variability of isoflavone content across different feed ingredients commonly used in livestock nutrition.

Moderate consumption of traditionally prepared and minimally processed soy foods may offer modest health benefits while minimizing potential adverse effects [23]. Soybean isoflavone plays a crucial role in anti-cancer, cardiovascular disease prevention, osteoporosis, and menopause syndrome, and can be used as a green and effective feed additive for animals [24]. Soybean isoflavones, mainly genistein, daidzein, and glycitein, have estrogen and anti-estrogen effects, and are widely used in animal feeding operations for their antioxidation, immune, and reproductive benefits [25]. Isoflavones, found in soybeans, can be used as alternative therapies for hormonal disorders, but may also be endocrine disruptors, posing potential health risks [26].

4. CONCLUSION

Isoflavones, which are phytoestrogens with numerous health benefits, were found in elevated levels in soybean meal compared to other livestock feed ingredients. This raises the possibility of transferring genistein and daidzein from feed into eggs, milk, and tissues, potentially leading to the production of designer eggs, milk, or meat enriched with isoflavones. Long-term clinical trials with standardized phytoestrogen preparations are needed to confirm these beneficial health effects.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Generative AI technology was used during the writing and editing of this manuscript. Specifically, ChatGPT 4.o was employed for paraphrasing and grammar checking. The input prompts provided to ChatGPT included requests for improving grammatical accuracy only.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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