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# Analysis of Phytoestrogens by High Performance Liquid Chromatography

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**Abstract**

Phytoestrogens are biochemicals synthesized in plants which mimic steroidal estrogen activity in mammals. Analysis of these compounds in the legumes which produce them and in body fluids is important to the study of their physiological effects. High pressure liquid chromatography (HPLC) has been found to be an efficient and sensitive method of identification and quantitation of isoflavonoids, one class of phytoestrogen. Here we report the separation of three isoflavonoids, biochanin A, genistein and daidzein using an HPLC system with a $C_8$ reverse phase column and a linear gradient mobile phase containing acetonitrile and acetic acid/water (10/90, v/v) over 60 minutes. Minimum detection limits for the three isoflavonoids were 0.556 µg/mL, 0.314 µg/mL, and 0.377 µg/mL, respectively. This method was used to measure the concentrations of isoflavonoids in two types of soy meal and in several animal feeds. Projected use of this assay includes studies of reproductive ability following ingestion of these isoflavonoids in domestic ruminants and in wild rodents.

## Introduction

Recently, much interest has focused on environmental endocrine disruptors, compounds available in the environment which interfere with the natural balance of hormones in animals which ingest them (Campbell and Hutchinson, 1998). Many of these compounds have been found to mimic the activity of natural estrogen and, therefore, effect reproduction (European Commission, 1996). One class of agents which has these effects is the phytoestrogens. As their name implies, phytoestrogens are produced by plants and have estrogenic activity in herbivorous animals. Isoflavonoids are a group of phytoestrogens which share a similar 3-phenyl chroman molecular structure (Katzenellenbogen, 1995; Miksicek, 1995) and which are produced in legumes, particularly soybeans and clovers, as well as several other types of plants (Phillips, 1992). The estrogenic effects of isoflavonoids have been studied in humans who consume soy and in ruminants that ingest phytoestrogenic clovers. In the 1940's, ewes grazing on estrogenic clovers suffered both permanent and temporary infertility (Bennets et al., 1946) accompanied by histological changes in cervical and uterine tissues, as well as ovarian failure and changes to other estrogen-sensitive tissues (Cheng et al., 1953; Lightfoot et al., 1967; Adams, 1977, 1981; Nwanenna et al., 1995). Recent studies have focused on phytoestrogens' effects on the developing fetus and gestation success, and on hormone-dependent tumors, such as breast cancers (Faber and Hughes, 1993; Medlock et al., 1995; Lamartiniere et al., 1998; Adlercreutz, 1998).

The isoflavonoids include the two parent compounds, biochanin A and formononetin (Fig. 1), which exist as glycoside conjugates in the plant (Harborne, 19731). These compounds are readily hydrolyzed by plant or digestive enzymes or by microorganisms in the rumen and further metabolized to the demethylated compounds, genistein and daidzein (Lundh, 1995) (Fig. 1). Genistein has been found to be quite estrogenic in several species (Miksicek, 1995) and inhibits tyrosine phosphokinase (Akiyama et al., 1987), an important second messenger system in several cell types including gametes and tumor cells (Moore and Kinsey, 1995; Linassier et al., 1990; Burks et al., 1995). Quantitation of phytoestrogens in foods and in animal tissues and fluids is crucial to future investigation of these agents.

Several techniques have been developed to separate and quantitate phytoestrogens including radioimmunoassay (Wang, 1998), gas chromatographic/mass spectrometric methods (GC/MS) (Adlercreutz et al., 1995), and high performance liquid chromatography (HPLC) (Lagana and Marino, 1991; Franke and Custer, 1994). Radioimmununoassay techniques developed by Wang (1998) are highly sensitive, but limited to only one compound, and may produce false positive results (Lagana and Marino, 1991). GC/MS is also sensitive, but it is time consuming and demands expensive instrumentation and intensive analytical experience (Franke et al., 1995). HPLC has proven to be an excellent separation, purification and detection technique (Lagana and Marino, 1991); it requires fewer preparatory steps than GC/MS and less expensive equipment. Most HPLC assays for phytoestrogens have

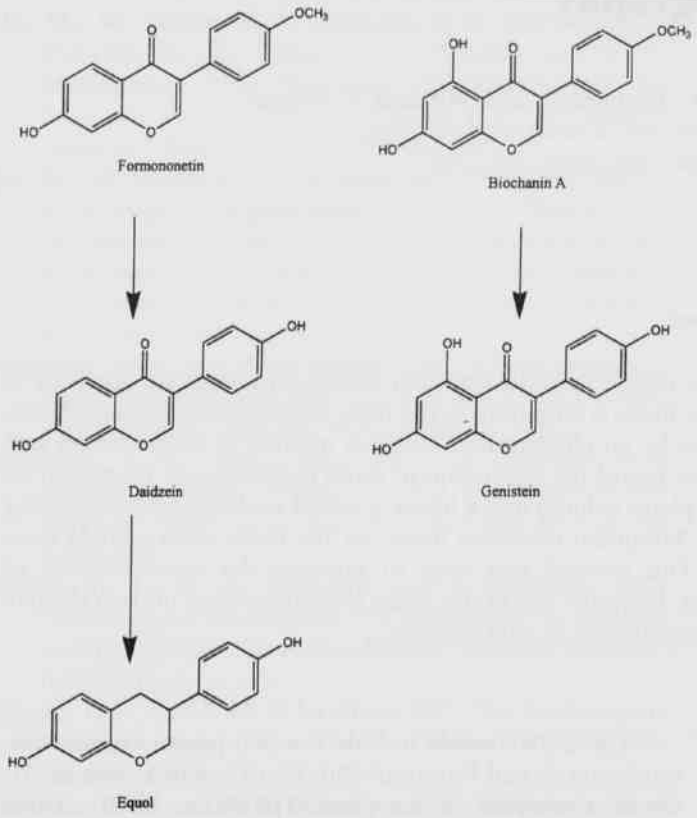


Fig. 1. Structures of parent phytoestrogens analyzed and their metabolites.

employed a $C_{18}$ reverse phase column (Gildersleeve et al., 1991; Coward et al., 1993; Franke and Custer, 1994; Wang and Murphy, 1994) with various mobile phases including acetonitrile and trifluoroacetic acid, methanol and ammonium acetate, and acetonitrile and ammonium formate buffers in different ratios and with various elution programs.

Here we describe an HPLC method employing a $C_8$ reverse phase column with a linear gradient mobile phase of acetonitrile and acetic acid/water. This system successfully separated one parent isoflavonoid, biochanin A, and two metabolites, daidzein and genistein, and was used to quantitate these compounds in two types of soy meal and three animal feeds.

## Materials and Methods

All solvents used for HPLC and optical density readings were of analytical or HPLC grade, vacuum-filtered through a 47 mm 0.45 µm nylon 66 membrane filter (Alltech Associates, Inc., Deerfield, IL). Methanol and HPLC grade water were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ. Acetic acid was purchased from EM SCIENCE, Gibbstown, NJ, and acetonitrile was from Fisher Scientific Company, Fair Lawn, NJ. Daidzein, genistein and biochanin A standards were purchased from Sigma Chemical Co., St. Louis, MO.

A Dionex 500 HPLC system with a 25 µL injector loop, in-line vacuum degasser, and AD20 absorbance detector was used in this study. An Adsorbosphere $C_8$ direct-connect guard column was coupled through a 2 µm in-line filter to a Zorbax SB-$C_8$ analytical column (4.6x15 cm, particle size 5 µm; MAC-MOD Analyticals, Inc., PA). Dionex PeakNet software controlled the instruments and automatically collected, processed, and reported the data.

All samples were injected individually through a syringe filter (25 mm diameter PTFE membrane, 0.2 µm pore size, Supelco, Inc., Bellefonte, PA). Elution was performed at 1 mL/min with the following stepwise gradient over 60 minutes. Where A=acetic acid/water (10/90, v/v) and D=acetonitrile, 30%D in A (v/v) ran for 20 minutes; from 20 minutes to 35 minutes, D changes from 30% to 70% and A changes from 70% to 30%; finally 70% D ran for 25 minutes. Phytoestrogens were detected by UV absorbance at 260 nm. A methanol rinse followed each run. Standard phytoestrogen solutions were prepared by addition of methanol to purified phytoestrogen, yielding stock solutions of 10 mg/mL, which were stored in amber vials at <-20°C. Stock solutions were then diluted with methanol to six different concentrations (0.5-100 µg/mL), and triplicate HPLC analyses were performed for each concentration to produce calibration curves for each phytoestrogen. This allowed calculation of the correlation coefficient, response factor, and minimum detection limit for each compound tested.

Phytoestrogens were extracted from two types of soy meal, hull-less and containing hulls (the generous gift of Mr. L. Gringas of Riceland Foods, Stuttgart, AR), and three animal feeds (Bermuda and fescue grasses and cottonseed) by a modification of the method of Franke et al. (1995). Briefly, 10 g of dry meal or chopped feed was finely dispersed in a mixture of 100 mL 77% ethanol containing 2M HC1 and 0.05% BHT as an antioxidant with stirring and sonication for 10 minutes. The solution was then refluxed (boiling point=78.5°C) for 2 hours, cooled to room temperature, and centrifuged at 850xg for at least 10 minutes. The clear supernatant was injected through a syringe filter into the HPLC system.

To quantitate any degradation of the phytoestrogens by the extraction procedure, new phytoestrogen standard solutions were prepared using ethanol as solvent and extracted in 77% ethanol/2 M HC1/0.05% BHT with stirring, sonication and reflux then analyzed by HPLC identically as the meal and feed samples had been. Also, the solution used for extraction was analyzed alone to ensure that it contained no constituents with interfering UV absorbances.

Table 1. Retention times, coefficients of determination, response factors and minimum detection limits for phytoestrogens from HPLC analyses.

| Phyto-estrogen | Retention Time (t) (+/- 0.5 min) Unex'd Std. | Ext'd Std. | Coefficient of Determination ($r^2$) Unex'd Std. | Ext'd Std. | Response Factor | Minimum Detection Limit (µg/mL) |
|---|---|---|---|---|---|---|
| Daidzein | 6.12 | 5.72 | 0.99972 | 0.99975 | $5.90 \times 10^{-5}$ | 0.377 |
| Genistein | 9.03 | 8.15 | 0.99986 | 0.99617 | $7.72 \times 10^{-6}$ | 0.314 |
| Biochanin A | 25.02 | 21.77 | 0.99991 | 0.97052 | $7.76 \times 10^{-6}$ | 0.556 |

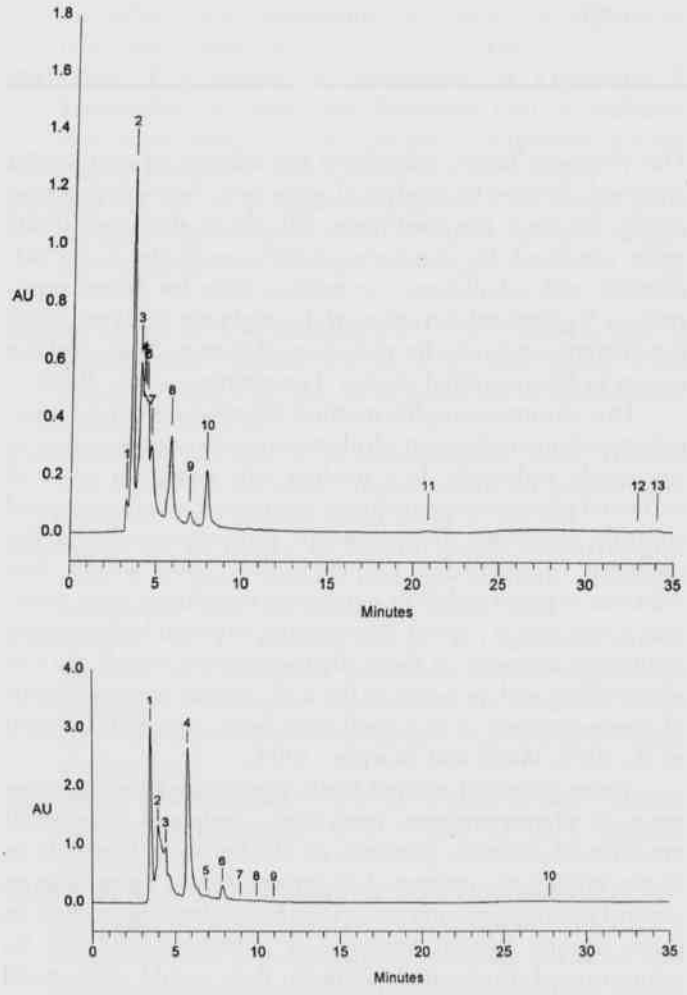


Fig. 2. Chromatographs of phytoestrogens extracted from soy meals by reflux in ethanol/HC1/BHT. (A) Hull-less soy meal extract. Peak identification: 8=daidzein; 10=genistein; peaks 1-7 and 9,11-13 are unknown compounds. (B) Extract of soy meal with hulls. Peak identification: 4=daidzein; 6=genistein; peaks 1-3 and 5,7-10 are unknown compounds.

## Results

The elution order for the three phytoestrogens analyzed is daidzein, genistein, biochanin A. Retention time for each phytoestrogen, correlation coefficients, response factors and minimum detection limits were determined from calibration curves for each phytoestrogen, and are summarized in Table 1.

When pure extraction solution (77% ethanol containing 2 M HC1 and 0.05% BHT) was refluxed and analyzed by HPLC, three peaks were identified at retention times of 3.4, 3.5 and 37.23 minutes, which did not overlap with retention times for any phytoestrogen extracted in this solution by this method. Extraction of pure phytoestrogen solutions by stirring, sonication and reflux in ethanol/HC1/BHT yielded recovery of 99.1% daidzein, 49.7% genistein and 93. 0% biochanin A. Mean retention times shifted slightly to the left for each phytoestrogen following extraction (Table 1).

Both hull-less soy meal and soy meal containing hulls contained measurable amounts of daidzein and genistein, but no detectable biochanin A, following their extraction in 77% ethanol containing 2 M HC1 and 0.05% BHT (Fig. 2). Soy meal with hulls contained considerably greater amounts of both daidzein and genistein than hull-less soy meal (Table 2).

Following extraction of phytoestrogens from Bermuda and fescue grasses and cottonseed, daidzein was not detected, but all feeds were found to contain genistein. Bermuda grass showed the highest concentrations of both genistein (2.8% of total peak area) and biochanin A (3.9%). Fescue also contained both of these phytoestrogens, but at less than 0.05% of its total constituents. Cottonseed contained only genistein (0.2% of its constituents). The major peak (91.8% peak area) in the Bermuda grass extract was found at t=4.03 min; the largest HPLC peak in the fescue grass extract (93.9%) occurred at t=12.8 min; and in the cottonseed extract a triplet peak occurred at t=4.03/4.62/4.77 min comprising 86.6% total peak area. None of these appeared to

Table 2. Concentrations of daidzein and genistein in soy meals.

| | Hull-less Soy Meal | | Soy Meal with Hulls | |
|---|---|---|---|---|
| | Daidzein | Genistein | Daidzein | Genistein |
| Peak area | $4.87 \times 10^6$ | $3.51 \times 10^6$ | $5.53 \times 10^7$ | $8.83 \times 10^6$ |
| Sample Concentration (µg/mL) | 54.0 | 25.9 | 612.2 | 65.2 |
| Recovery Percentage | 0.991 | 0.497 | 0.991 | 0.497 |
| Phytoestrogen Concentration (µg/mL) | 54.5 | 52.2 | 617.8 | 131.2 |

overlap with retention times for daidzein, genistein or biochanin A.

## Discussion

The HPLC method developed here allows rapid, precise and inexpensive analysis of daidzein, genistein and biochanin A and is consistent with elution orders of previous HPLC research of similar compounds (Franke and Custer, 1994; Coward et al., 1993; Gildersleeve et al., 1991). While most earlier HPLC separations of phytoestrogens utilized $C_{18}$ columns (e.g., Wang and Murphy, 1994), the results of this study showed that a $C_8$ column, in combination with an acetonitrile/acetic acid elution system, is also efficient in separating the three compounds. Retention times in both types of columns were similar, though use of the $C_8$ column seemed to maintain a more stable baseline throughout the run, resulting in enhanced resolution of later peaks, i.e., the biochanin A peak. Also, earlier methods often employed DMSO as solvent for these hydrophobic compounds. However, DMSO apparently interfered with the PEEK tubing in our system, effecting retention times (pers. comm., Dionex Corp.). Better results were obtained when methanol was used to dissolve the samples, avoiding the influence of DMSO.

The correlation coefficient ($r^2$) provides a measure of the quality of curve fit of the calibration curves, with $r^2=1$ correlation a perfect linear fit. Calibration curves for each phytoestrogen, either standard solution directly or following extraction in ethanol/HC1/BHT, showed $r^2>0.996$ with the exception of biochanin A following extraction ($r^2=0.971$). The response factor, calculated by amount of compound analyzed divided by analytical response, shows strong sensitivity for each phytoestrogen. Minimum detection limits were obtained by analyzing seven replicate 1 µg/mL aliquots and calculating t (retention time for seven replicates) x S (standard deviation of the replicate analyses). This is sufficient sensitivity for detection of dietary doses of these agents in future animal studies (Lamartiniere et al., 1998).

This chromatographic method afforded successful separation and quantification of phytoestrogen concentration in soy meals and feeds. It is unclear why retention times of extracted phytoestrogens shifted compared to non-extracted controls, nor why the extraction procedure reduced the recovery of genistein. Earlier studies have shown that daidzein is more stable to extraction conditions than genistein (Franke et al., 1995). Apparently, soybean hulls contain significant amounts of these phytoestrogens, which are lost after milling with removal of the hull. Similar concentrations of phytoestrogens in soy meal have been reported (Coward et al., 1993; Wang and Murphy, 1994).

Three potential animal feeds were tested for the presence of phytoestrogens, and none contained substantial amounts of daidzein, genistein or biochanin A. Bermuda or fescue grasses or cottonseed, therefore, would be suitable as control or supplementary diets for future feeding studies. In these studies, known amounts of phytoestrogens will be administered to treated animals that would also need supplemental diets containing feeds which would not supply additional phytoestrogens.

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