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# Soybean isoflavones: estrogenic activity and isolation of two new isoflavones

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**SOYBEAN ISOFLAVONES: ESTROGENIC ACTIVITY AND ISOLATION OF  
TWO NEW ISOFLAVONES**

*Iowa State University*

Ph.D. 1984

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**Soybean isoflavones: Estrogenic activity and isolation of two new  
isoflavones**

by

**Efi D. Farmakalidis**

**A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
Requirements for the Degree of  
DOCTOR OF PHILOSOPHY**

**Major: Food Technology**

**Approved:**

Signature was redacted for privacy.

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## DEDICATION

To my parents who made my dream come true.

## INTRODUCTION

The term flavonoid is generally used to denote the group of plant phenols characterized by the carbon skeleton C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub>. The basic structure of these compounds consists of two aromatic rings linked by a three carbon aliphatic chain which normally has been condensed to form a pyran or less commonly a furan ring. Based chiefly on the oxidation state of the aliphatic fragment, these compounds may be subdivided into several groups; the flavones, flavanones and isoflavones. Figure 1, shows the different types of flavonoids and a brief description of the class.

## Chemistry of Flavonoid Compounds

The isoflavones are one of the most difficult classes of flavonoids to characterize, since they do not respond specifically to any color reaction. Unlike many other flavonoids, however, isoflavonoids are usually very easily crystallized from plant extracts and as a class lend themselves particularly well to the classical techniques of organic chemistry.

Probably the most useful tool in the identification of flavonoids is the use of ultraviolet (UV) spectroscopy. All flavonoids have intense absorption in the short UV (ca 210nm), an intense band at 255-275nm and generally a less intense band or inflection at 320-330nm. In isoflavones, the phenyl ring at position 3 is not in conjunction with

the pyrone carbonyl as it is in the flavones so that the spectral properties differ significantly from those of flavones. A distinctive feature of the isoflavones is their low intensity of absorption above 300nm. Isoflavone spectra undergo bathochromic shifts in the presence of alkali. Such measurements are often of value in structural studies. The presence of a free 5-hydroxyl group in an isoflavone can be detected by means of measuring the effect of ethanolic aluminium chloride on the spectrum. The presence of a free 7-hydroxyl can be detected by measuring the effect of adding sodium acetate to the solution. In both instances, the maxima undergo bathochromic (i.e. towards a longer wavelength) shifts of 10-15nm .

Isoflavones can be detected on paper or silica gel with a general phenolic reagent (e.g.  $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$  or Folin), but there does not seem to be a more specific spray available. However, 5-deoxyisoflavones (e.g. daidzein, afrormosin) have a brilliant light blue fluorescence in UV light and ammonia. Genistein and other 5-hydroxyisoflavones appear as dull purple spots in UV light, changing to dull brown with ammonia.

The methods of extraction of the isoflavonoids differ depending upon the characteristics of the plant source and particularly upon the impurities present. For example, seeds are frequently rich in oils, waxes and proteins. Leaves contain a great deal of resin, wax and chlorophyll. The solvents normally used for extraction are alcohol, acetone, ether and light petroleum. In some cases, water can be used but this is unusual because water introduces other impurities in the extract such as phospholipids.

Methods currently in use for the separation and purification of the flavonoids include paper chromatography, thin-layer chromatography and column chromatography utilizing adsorbents such as alumina (Naim et al., 1974) silica gel and Sephadex LH-20 (Ohta et al., 1979). Gas liquid chromatography has also been used on a preparative scale. However, its use is limited to volatile samples (Preston, 1966, pp.35-40). In the isoflavonoid field, high-performance liquid chromatography (HPLC) has been mainly used as an analytical technique. Several papers have reported the use of reverse phase C<sub>18</sub> high-performance liquid chromatography in the separation and quantitation of the isoflavonoids in soybeans and soybean products (Eldridge, 1982a; Murphy, 1981, 1982). Isoflavonoids in forages have been assayed by various workers (Nicollier and Thompson, 1982; Patroni et al., 1982). The use of preparative high-performance liquid chromatography for isolation and purification of isoflavones has not been investigated.

Flavonoids have been reported to exhibit, antioxidant, antifungal and estrogenic properties.

#### Antioxidant Activity of Flavonoids

Many of the flavonoids and related compounds have strong antioxidant characteristics in aqueous lipid and lipid food systems. The major value of flavonoids is their primary antioxidant activity, i.e., as free radical acceptors and as chain breakers. Rutin and related flavonones were reported to decrease the fragility of blood

capillaries in guinea-pigs and these substances were once thought to possess vitamin-like activity in humans. The claims that rutin and related flavonones are "Vitamin-P" substances have never been substantiated. However, rutin (in massive doses) has pharmacological activity as an antioxidant towards adrenaline and ascorbic acid (Zlotch et al., 1977, pp. 445-454). Rutin also relaxes smooth muscle and acts as an inhibitor of enzymes. The isoflavones, genistein and daidzein, have been reported to exert an antihemolytic effect on erythrocytes subjected to peroxidation (Naim et al., 1976). Flavonoids are also effective against lipoxygenase catalyzed reactions. The compound 6,7,4'-trihydroxyisoflavone has been identified as the major antioxidant in fermented soy beans (Gyorgy et al., 1964). In addition to antioxidant effects Naim et al., (1974) reported that genistein and daidzein exhibit antifungal activity. Soybean products have also been reported to exhibit hypocholesterolemic effects. Recent reports attribute this hypocholesterolemic (cholesterol-lowering) activity of soy protein to the isoflavonoid constituents (Anonymous, 1980). However, the physiological significance of isoflavones in the diet as related to hypocholesterolemic effects requires further experimentation.

#### Estrogenic Effects

By definition, an estrogen is a substance capable of stimulating the growth of the vagina, uterus and the mammary gland and is responsible for the development of female secondary characteristics. Both natural

and synthetic estrogens have a wide variety in structure. The natural estrogens of animal origin are steroids with the cyclopentanoperhydrophenanthrene nucleus. The synthetic estrogens and those occurring in plants are not steroids, but possess similar biological characteristics to the naturally occurring animal estrogens. Many flavonoids have a benzopyran ring structure. The phytoestrogens which have an isoflavonoid structure are the most closely related to diethylstilbestrol (DES) and estradiol. The structural relationships between isoflavones, natural estrogens and synthetic estrogens is illustrated in figure 2. This structural similarity is the probable cause of the ability of isoflavones to mimic the estrogens in mammalian species.

More than 50 species of plants have been reported to contain differing degrees of estrogenic activity (Bradbury and White, 1954). The estrogenic principles in plants have been, in most cases, chemically identified as isoflavones which occur naturally in the form of glucosides. The sugar moiety is attached to one or more of the hydroxyl groups located at various positions of the isoflavone nucleus. Among the compounds with estrogenic activity that have been isolated and characterized from plants include the isoflavones, genistein, daidzein, biochanin A, formononetin and pratensein and the coumestans, coumestrol and 4'-methoxy-coumestrol (Bickoff et al., 1969). The estrogen content of plants has been reported to be influenced by environmental factors. Alexander and Watson (1951) found considerable seasonal variations in the estrogenic activity of subterranean clover. Recently, Eldridge and

Kwolek (1983) also reported variations in the isoflavonoid constituents in soybeans grown under different environmental conditions. It is of interest to note that when plants are infected with fungus, bacteria or viruses or attacked by aphids or leaf hoppers, the concentration of phytoestrogens increases (Loper and Hanson, 1964; Francis and Millington, 1971). Since many pasture plants are preserved for winter feeding to stock, it is of practical interest to determine whether the processes used to preserve the plants alter their estrogenic activity. During fermentation of pasture plants, the original "estrogenic" compound becomes modified to give a more active substance (Pieterse and Andrews, 1956a, b). In contrast, it was observed that the drying of clover leads to a loss in its estrogenic activity (Alexander and Watson, 1951).

Most of the information available on the estrogenic effects of the isoflavones has been mainly concerned with the adverse effects they have on the reproductive system of sheep. Phytoestrogens have been found to cause serious infertility in grazing sheep and possibly also in cattle (Moule et al., 1963; Bickoff, 1968; Braden and McDonald, 1970, pp. 381-91). The isoflavones, genistein and daidzein, isolated from subterranean clover were found to be the active factors responsible for interference in the estral cycle of sheep that caused serious reduction in lambing percentages (Bennets et al., 1946). The infertility caused in both sheep and cattle was a considerable agricultural problem, which has been solved in part by restricting the intake of clover during the breeding season. An effort was also made to produce clover strains with



little or no isoflavone content (Francis and Millington, 1965). The isoflavone content of clover fodder, on the other hand, was shown to increase the rate of growth of fattening stock and also have a beneficial effect on lactation in cows, contributing to the so-called "spring flush" in milk yield.

Genistein and daidzein are roughly  $10^5$  times less effective estrogens, as shown by uterine enlargement assays, than DES in mice (Bickoff et al., 1962; Millington et al., 1964). It takes about 8mg genistein and 11mg daidzein to produce uterine enlargement in mice equivalent to  $0.08\mu\text{g}$  of DES. Genistein and coumestrol, have been shown through competitive binding assays to bind to estrogen receptors using rabbit (Shemesh et al., 1972) sheep (Shutt and Cox, 1972) and rat (Verdeal, 1978) uterine cytosol. It is probable that the phytoestrogens, bind to the hypothalamic and hypophyseal tissues as well, following the pattern of the steroidal estrogens. The steroidal estrogens, naturally found in mammalian organisms, bind in the hypothalamus and the pituitary. This binding exerts a negative feedback inhibition on estrogen release by the ovaries and completes the hypothalamo-hypophyseal-gonadal interrelationship. It has been suggested that the infertility in clover affected ewes was due to an inability of target organs to give a continued response to the "priming" action of estrogen (Adams, 1979). Furthermore, it was suggested (Findley et al., 1973; Adams, 1979) that the prolonged exposure to plant estrogens causes persistent desensitization of the hypothalamus of the ewe to estrogen. Newsome and Kitts (1977) suggested that the plasma

levels of phytoestrogens resulting from the consumption of alfalfa could be high enough to compete with endogenous estrogens for sites on the estrogen binding protein in the hypothalamus of the ewes thus influencing the production of gonadotropins and depressing the functioning of the ovary.

Recent reports (Kitts et al., 1980) investigating the similarity between endogenous estrogens and phytoestrogens in their mode of action have shown that common feedstuffs containing phytoestrogenic activity influence biochemical processes preceding net increases in target tissue synthesis similar to changes occurring with natural estrogens. Receptor complexes of coumestrol and genistein were found to translocate to the nucleus and were retained for at least 25 hours at levels similar to those found after injection of 17- $\beta$ -estradiol (Newsome and Kitts, 1980). In contrast, the phytoestrogens have also been reported to act as antagonists to endogenous estrogen. Antagonistic compounds generally compete for 17- $\beta$ -estradiol receptors and fail to stimulate the nucleus to respond fully (Anderson, et al., 1972). In view of the fact that phytoestrogens have been shown to bind with relatively high affinities to the estrogen receptors of human mammary tumor cells (Martin et al., 1978; Verdeal et al., 1980) their effects as antiestrogens could have a beneficial effect as related to mammary tumors.

Estrogens in pasture legumes have been assayed by various workers using mice, guinea pigs and sheep (Davies and Bennet, 1962; Morley et al., 1968). Early studies suggested that phytoestrogens differ in potency among animal species. In sheep, the estrogenic activity of

different varieties of clover was correlated with the formononetin content, but not with the content of genistein or biochanin A (Braden et al., 1967). In contrast, formononetin was found to be inactive or much less active than genistein when fed to mice (Bickoff et al., 1962). The high estrogenic activity of formononetin compared to genistein in sheep has been shown to be a direct result of the metabolism of the two isoflavones. Genistein is degraded by microorganisms in the rumen to give non-estrogenic products. Degradation of formononetin in the rumen gives daidzein and equol, an estrogenically active compound in sheep (Batterham et al., 1971; Shutt et al., 1970). The relative estrogenicity of genistein and formononetin in sheep is, therefore, the opposite of that in mice. The metabolic pathways to inactivate estrogenic isoflavones, then, can have a decisive effect on the overall significance of the presence of these substances in foods. Animal species were also found to differ in the sensitivity to phytoestrogens with sheep being more sensitive than mice (Morley et al., 1968).

Unfortunately, the majority of the information available on plant estrogens is concerned with estrogenic substances in feeds (clover, alfalfa, fungus infected grains), rather than concerned specifically with those estrogens of significance in human foods. Among the plants commonly used for human food, the following have been reported to contain substances that elicit an estrogenic response in an appropriate experimental animal: carrots (Ferrando et al., 1961), soybeans (Carter et al., 1955) wheat, rice, oats, barley, potatoes, apples, cherries, plums (Bradbury and White, 1951) wheat bran, wheat germ, rice bran and

rice polish (Booth et al., 1960). Proteins derived from plant sources are gaining importance as part of the diet replacing animal proteins; soybeans are a major source of human protein. The isoflavones daidzein, daidzin, genistin and genistein have been isolated from soybeans (Carter et al., 1955; Nilson, 1962; Naim et al., 1974). Genistin has also been isolated from commercial soybean meal that has presumably been heated (Magee, 1963) showing that this substance is stable to the heat treatment involved in the toasting process. Recent reports (Drane et al., 1980) have drawn attention to the fact that rat chow used as a control feed in routine mouse bioassays for estrogens had developed significant uterotrophic activity over a period of a few months. The estrogenic activity was attributed to soy meal which made up 10% of the rat chow. The estrogenic effect of soybean meal was attributed to the presence of genistein and daidzein. However, 95-99% of the isoflavone content of defatted soybean meal (Naim et al., 1974) and of whole soybeans (Murphy, 1982) is in the form of the glucosides. The aglucones, genistein and daidzein, occur naturally only in low concentrations. Very little information exists on the possible adverse effects genistin and daidzin might have. Their estrogenic potency has not been investigated. In view of the fact that there is a significant carryover of these forms into soy-protein foods intended for human use, (Murphy, 1982) it is important that more information be obtained concerning these compounds.

Several other isoflavones have recently been reported to be present in defatted soybean meal and defatted soybeans. Naim et al. (1973;

1974) has reported the presence of glycitein-7- $\beta$ -0-glucoside and glycitein, methoxy derivatives of daidzin and daidzein respectively. Glycitein-7- $\beta$ -0-glucoside has also been reported by Eldridge (1982a, b) in defatted soybean meal. These compounds however, have not been detected and their structure has not been chemically confirmed by other researchers. Ohta et al. (1979, 1980) have reported the isoflavones 6"-0-acetyldaizidin and 6"-0-acetylgenistin to be present in defatted soybeans. Murphy (1982) observed the presence of two extra peaks in chromatograms of some foods such as textured vegetable protein (TVP) which were not present in chromatograms of untreated soyflour. One of the peaks increased in intensity with an increase in storage time of the product. The compounds contained in those peaks were not identified. The presence of glycitin and its aglucone, acetylgenistin and acetyldaizidin require further substantiation to classify these compounds as native soybean isoflavones, rather than products of processing or the method of extraction. Bioactivities for these compounds have not been reported. Further experimentation is required to determine the importance of these compounds in soybeans and/or soybean products, intended for human use.

#### Explanation of dissertation format

The dissertation is divided in five parts each being a complete paper already submitted (parts I, II and III) or to be submitted (IV and V) to professional journals. Part I examines the use of preparative high-performance liquid chromatography as an alternative, to

conventional chromatographic methods, currently in use, for the isolation and purification of isoflavonoids from soybeans. Parts II and III examine the response of different strains of mice, to the isoflavones genistein, daidzin, genistin and the synthetic estrogen diethylstilbestrol. Part IV, a continuation of the experiments reported in parts II and III reports the estrogenic potency of the isoflavones genistin and daidzin in the B6D2F1 strain of mouse. Part V reports the isolation and purification of two other isoflavonoids, 6"-0-acetyldaidzin and 6"-0-acetylgenistin.

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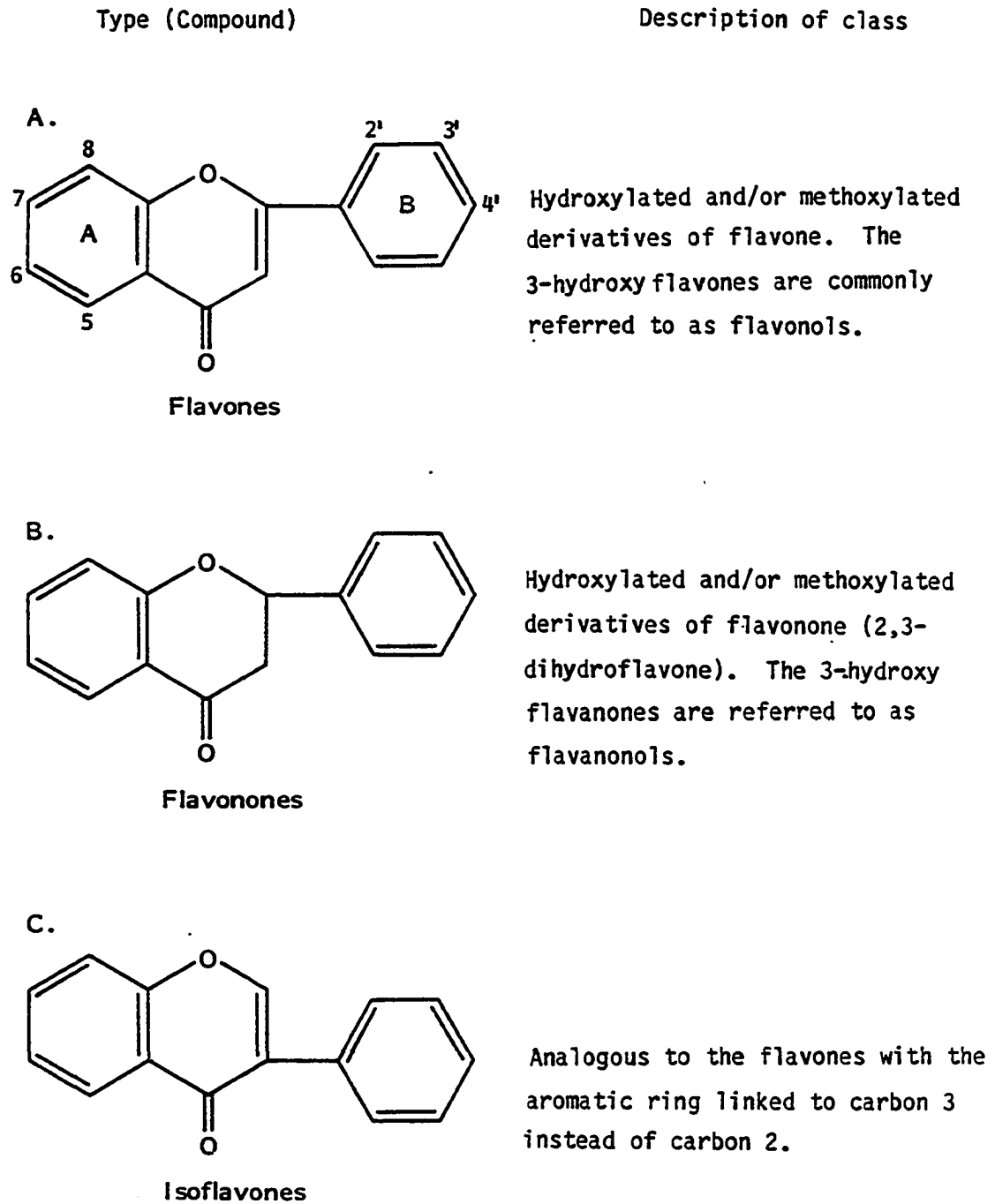


Figure 1. Types of flavonoids

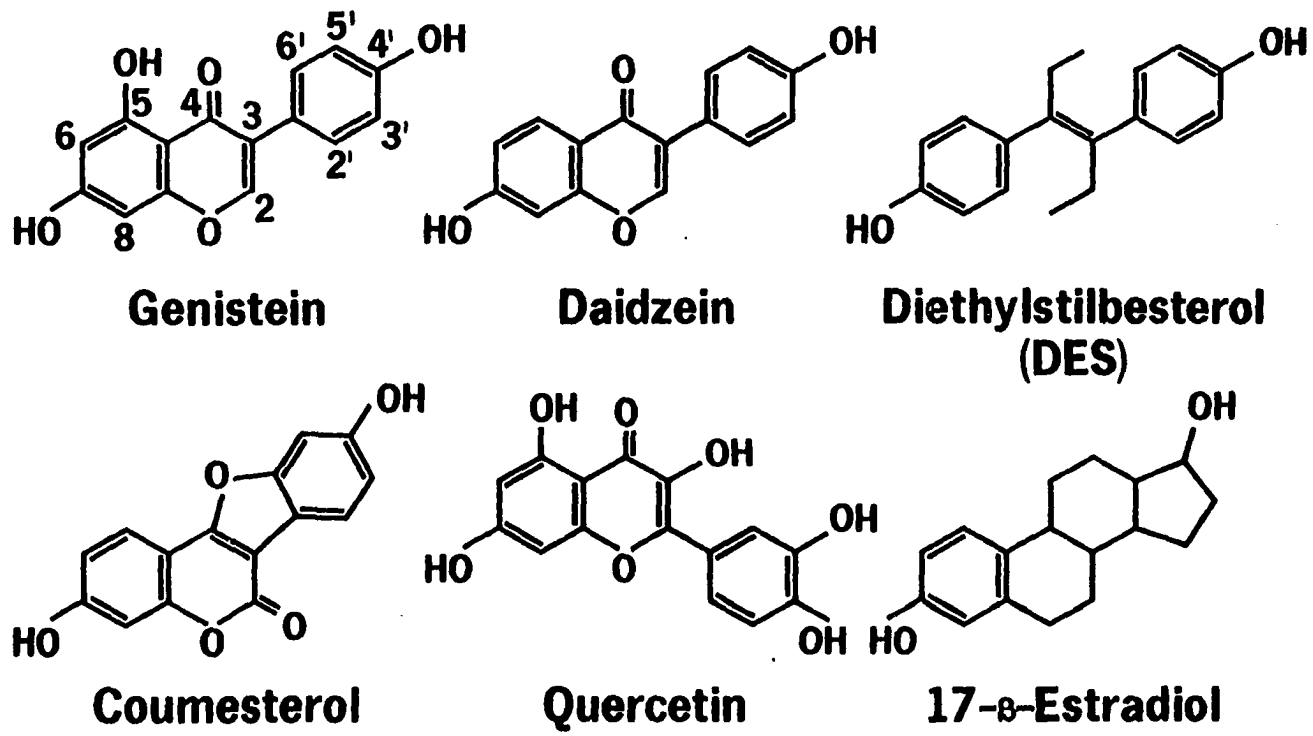


Figure 2. Structural comparison of flavonoids (flavones and isoflavones) and estrogens

PART I. SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC  
ISOLATION OF SOYBEAN ISOFLAVONES

by

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## ABSTRACT

Semi-preparative high-performance liquid chromatography has not been applied to the isolation and purification of the soybean isoflavones. Our paper reports a method for the isolation of these compounds. The compounds have a low solubility in the solvents suitable for reverse-phase chromatography, and this limits the amounts applied to the column. However, the method does offer several advantages over conventional methods already in use.

## INTRODUCTION

Soybeans are known to contain the estrogenic isoflavones genistein and daidzein; however, the glucosides genistin and daidzin are the major forms of the isoflavonoids in soybeans. Previous reports indicate significant carryover of these forms into soybean protein products intended for human use (Murphy, 1982; Eldridge, 1982a).

Methods currently in use for the separation and purification of the flavonoids include paper chromatography, thin-layer chromatography and column chromatography utilizing adsorbents such as alumina (Naim et al., 1974) silica gel and Sephadex LH-20 (Ohta et al., 1979). Gas liquid chromatography has also been used on a preparative scale however its use is limited to volatile samples (Preston, 1966, pp. 35-40). The direct analysis of non-volatile and thermally unstable phenolics is impossible.

It is noteworthy that in the isoflavonoid field, high-performance liquid chromatography (HPLC) has been mainly used as an analytical technique; e.g. for checking the purity of natural samples, for quantitative determination of plant constituents (Eldridge, 1982b; Murphy, 1982; Murphy et al., 1982; Nicollier and Thompson, 1982; Patroni et al., 1982) and for chemotaxonomic comparisons. The use of HPLC on a preparative scale has not been investigated. Our paper reports how HPLC can be used on a preparative scale for the isolation and purification of the soybean isoflavones, namely genistin and daidzin.



## MATERIALS AND METHODS

Toasted defatted soyflakes were obtained from A. E. Staley Manufacturing Co. (Des Moines, IA). The flakes were extracted overnight with acetone 0.1N HCl in the ratio of 5 to 1 (ml/g flakes). This solvent system was previously found effective in extracting the isoflavones and gives low extraction of other interfering material (Murphy, 1981). After filtration, the extract was concentrated by using a rotary evaporator at 40°C. The reddish brown residue was suspended in chloroform-methanol (9:1) and passed through a 2.5 x 50 cm silica gel column (60-80 mesh, Baker Chemical Co., Jackson, TN) equilibrated with chloroform methanol (9:1). The column was washed with 300 ml of chloroform-methanol (9:1). This gets rid of some of the contaminating phospholipids as well as most of the free forms of isoflavones; namely, daidzein and genistein. The isoflavones (mainly genistin and daidzin) were eluted from the column with 250 ml of chloroform-methanol (50:50). After the solvent was evaporated, the residue was redissolved in methanol, filtered through a 0.45- $\mu$ m filter, and fractionated by using semi-preparative high-performance liquid chromatography.

The chromatographic system consisted of a reverse-phase 9.4 x 250 mm Partisil ODS-3 C<sub>18</sub> (Whatman) semi-preparative column with a 2-cm Lichrosorb RP-18 guard column (Brownlee labs). Separation of the peaks was performed with a non-linear methanol-water gradient, utilizing a Beckman/Altex microprocessor/controller and two model 110A pumps. Monitoring of the peaks was achieved by utilizing a fixed-wavelength UV

detector (Beckman/Altex) at 254 nm equipped with a preparative flow cell. Combined flow rate was 5 ml/min. Samples were injected by using a 2-ml loop. Total chromatography time was 16.5 min. Peak areas were integrated by using a Varian CDS-111 computer.

Purity of the compounds collected with this method was determined by using silica gel thin-layer chromatography (solvent system: chloroform:methanol:water, 65:25:4), analytical high-performance liquid chromatography (Murphy, 1982), and melting points. The results of these experiments were compared with results obtained with compounds isolated by utilizing conventional chromatographic methods (Ohta et al., 1979).

## RESULTS AND DISCUSSION

A non-linear gradient of methanol-water was found the most suitable solvent system in separating the soybean isoflavones, as can be seen in Figure 1. Preliminary experiments showed that an isocratic elution of the compounds would not be effective for adequate separation of the compounds on a preparative column. An analytical method for separating the isoflavones developed earlier in our laboratory (Murphy, 1981) was used as a reference point for the development of the gradient used.

Preliminary experiments indicated that the crude soybean extract could not be fractionated by using HPLC directly, mainly because of the large concentration of phospholipids in the extract. This contamination of the sample did not present problems in the analytical method because the sample size used was small. On a preparative scale, however, this contamination made it necessary to employ an initial silica-gel purification step before fractionation using preparative HPLC. Retention times for daidzin and genistin were 8.2 and 10.5 min, respectively.

As can be observed on Figure 1, peak 1 consists of two compounds. Efficient resolution of the two peaks could not be achieved with the gradient used for fractionation. The compound eluting closely to daidzin was not identified but was believed to be glycitin. Several reports on the presence of glycitin in soybean extracts denote the elution time of the compound to be close to that of daidzin (Eldridge, 1982a, b). That the two peaks eluted so closely made it necessary to

employ the technique of "heart-cutting" (Snyder and Kirkland, 1979, p. 617) for collection of daidzin. The technique involves collection of the center portion of the peak of daidzin and ignoring the leading and tailing portions. This reduced the amount of daidzin that could be collected in the original fractionation. Recycling of the daidzin peak was necessary. We utilized a different methanol-water gradient to allow further separation of the two peaks and further purification of the fraction (Figure 2) collected.

Collection of the genistin peak was easier because it only contained one compound, but recycling was required to get rid of contaminating material. The methanol-water gradient was changed to suit the purification of the compound (Figure 3).

After recycling twice through the column, daidzin was crystallized from 50% methanol and genistin was precipitated out of a super-saturated solution of 100% ethanol. The results of the various tests performed on the compounds are shown on Table 1. Thin-layer chromatography revealed one spot for each of the compounds. Analytical high-performance liquid chromatography revealed only one peak. Melting points were identical with those reported in the literature (Mabry *et al.*, 1970, p.109). The main limitation of the method was the amount of mixture that could be applied to the column. The maximum amount that could be applied was only 10 mg of a mixture of genistin and daidzin. The maximum solubility of the compounds in methanol was only 5 mg/ml. The recovery of pure sample was 30% of the original sample fractionated.

The time required to purify large amounts of the compounds is

comparable to the time required for the final fractionation of a mixture of daidzin and genistin by use of conventional chromatographic (Ohta et al., 1979) methods (Table 2). Therefore, the method offers some advantages over the conventional methods of isolation because the purification steps required are fewer. Apart from the original purification of the sample through silica gel, no other purification was necessary before application of the sample on the column. Use of conventional methods requires several purification steps before fractionation of the mixture into its components, genistin and daidzin.

Even though the solubility of the compounds in methanol is low, the use of preparative high-performance liquid chromatography still is more rapid than the conventional chromatographic methods presently reported in the literature.

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Table 1. Comparison of properties of isoflavones isolated by using (a) conventional chromatographic methods and (b) HPLC method

Compound	TLC R <sub>f</sub> value	Melting point	Retention time with analytical HPLC
Genistin <sup>a</sup>	0.61	256±2°C	8.3
Daidzin <sup>a</sup>	0.51	230±2°C	7.4
Genistin <sup>b</sup>	0.60	256±2°C	8.3
Daidzin <sup>b</sup>	0.51	230±2°C	7.4

<sup>a</sup>Compounds isolated using conventional chromatographic methods.

<sup>b</sup>Compounds isolated using HPLC.

Table 2. Comparison of times required for separation of daidzin and genistin

	HPLC Method	Sephadex LH-20 fractionation
Maximum load	10 mg	50 mg
Time required	16.5 min	12 hr
Recovery	3 mg	45 mg



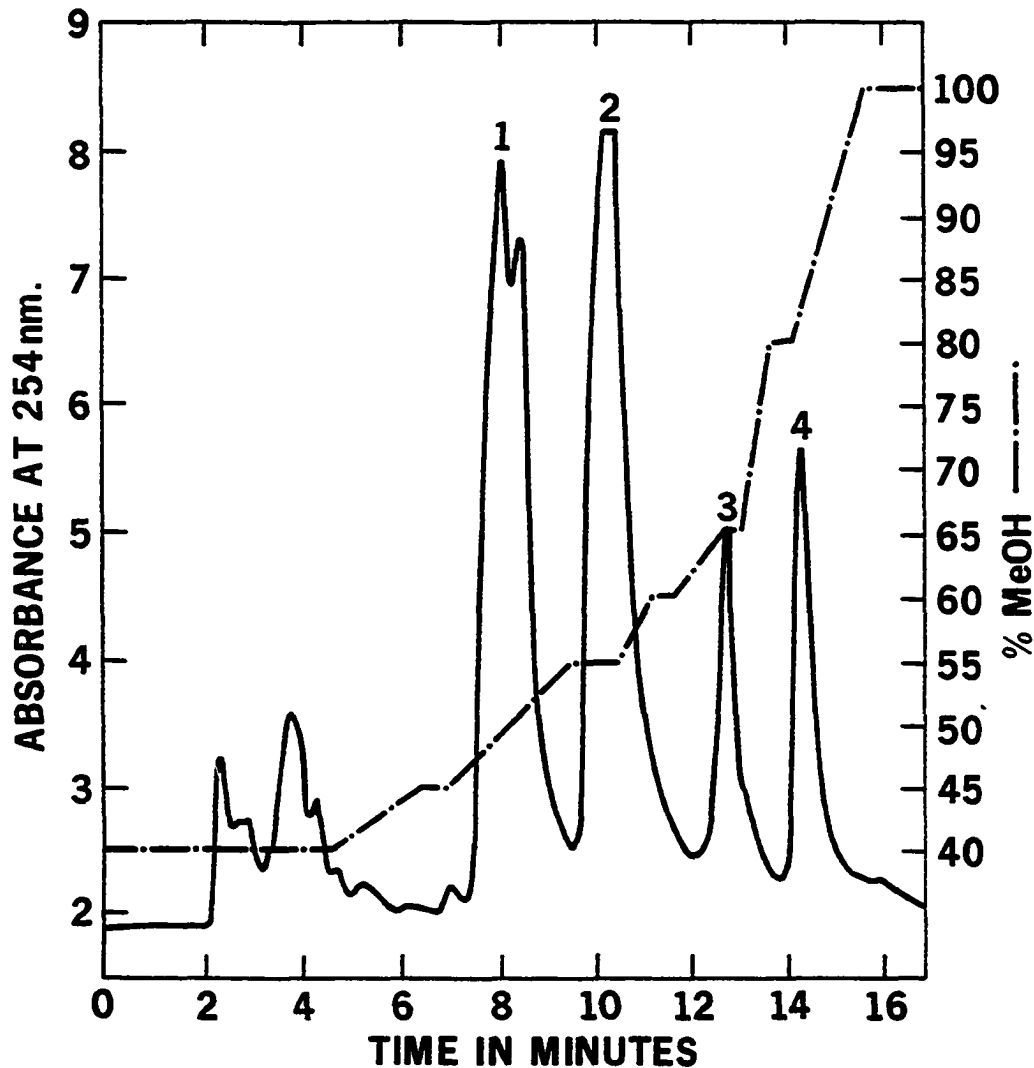


Figure 1. HPLC fractionation of crude soybean extract, on a reversed-phase  $C_{18}$  semi-preparative column (Whatman) utilizing a non-linear methanol-water gradient. The technique of "heart-cutting" was used for the collection of daidzin. Peaks: 1 = daidzin; 2 = genistin; 3 = daidzein; 4 = genistein. Each division on absorbance axis is equal to 0.4 absorbance

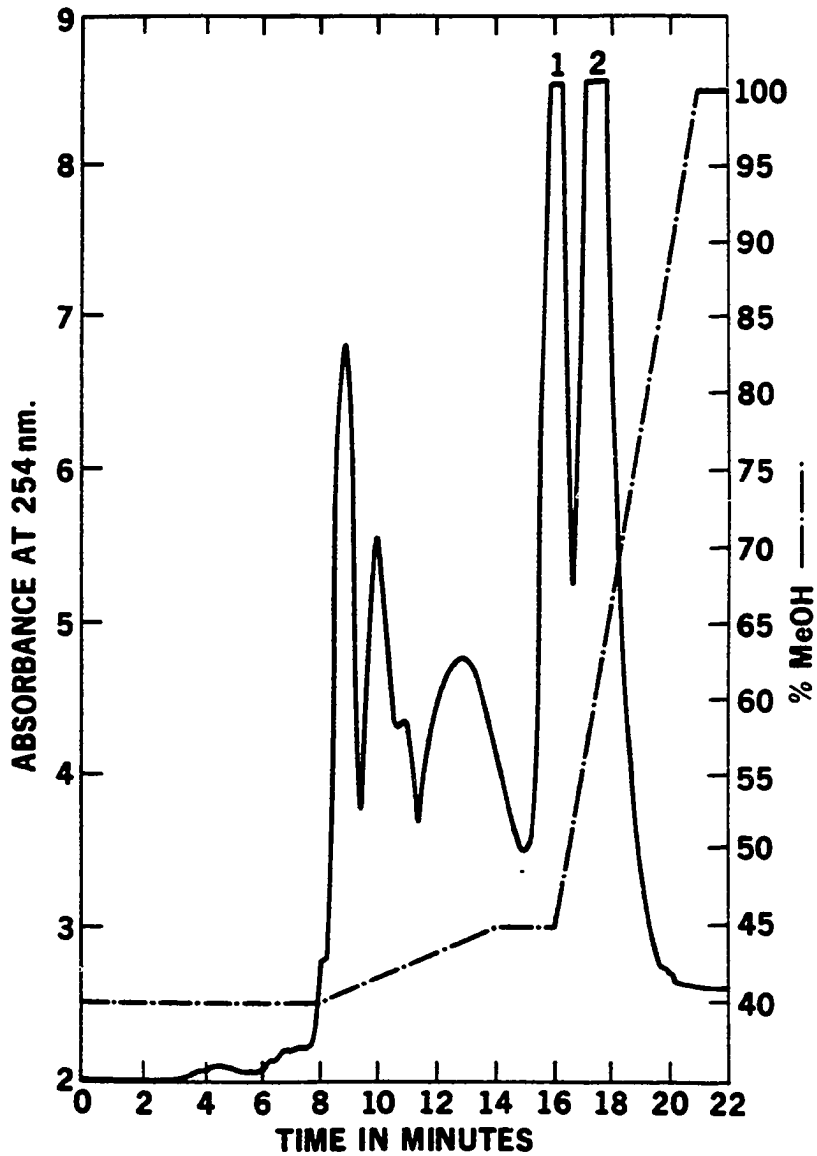


Figure 2. Purification of the daidzin peak collected by "heart-cutting", in Figure 1 and utilizing a new methanol-water gradient. Peaks: 1 = daidzin; 2 = not identified. Each division on absorbance axis is equal to 0.4 absorbance

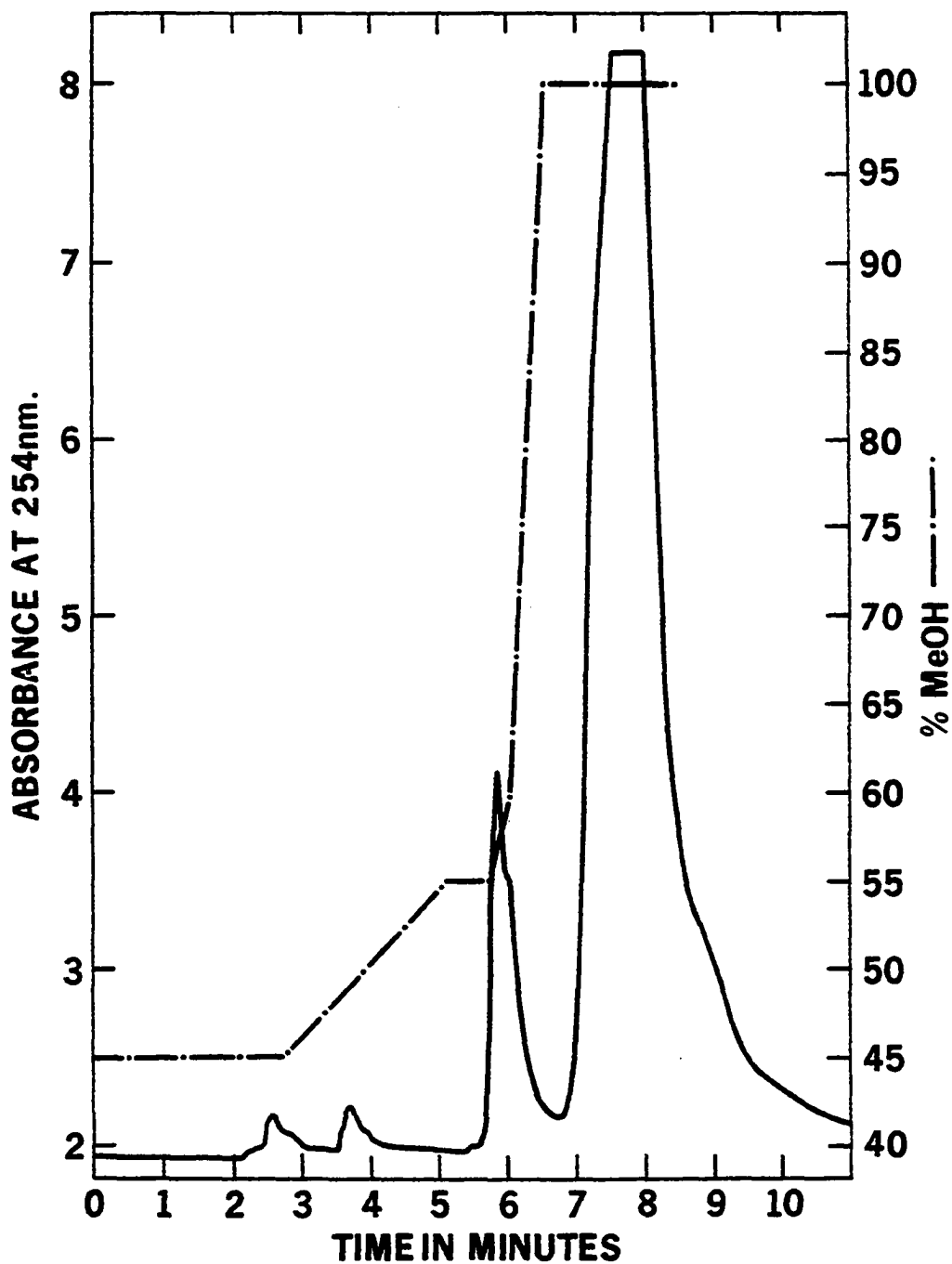


Figure 3. Purification of genistin fraction collected from Figure 1 and utilizing a different methanol-water gradient. Each division on absorbance axis equal to 0.4 absorbance

**ACKNOWLEDGEMENTS**

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PART II. ESTROGENIC RESPONSE OF CD-1 MOUSE TO THE SOYBEAN ISOFLAVONES  
GENISTEIN, GENISTIN AND DAIDZIN

by

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Running title: Estrogenic Response of CD-1 Mouse to Isoflavones

Submitted to: Food and Chemical Toxicology

## ABSTRACT

The soybean isoflavones, genistein and daidzein, have been reported to exhibit estrogenic activity in mice. There is a significant difference in response to these estrogenic compounds between strains of mice. The CD-1 mouse does not respond to these estrogenic compounds found in soybeans or to diethylstilbesterol at levels reported previously.

## INTRODUCTION

Many naturally occurring compounds exhibit estrogenic activity and are widely distributed in both plants and animals. Among the compounds that exhibit estrogenic activity are the soybean isoflavones, genistein and daidzein. The glucosides of genistein and daidzein are the major forms of isoflavones in soybeans. These compounds have not been assayed for their biological potency as estrogens. Estrogens in pasture legumes have been assayed by various workers using mice, guinea pigs and sheep (Davies and Bennet, 1962; Bennet et al., 1967; Morley et al., 1968). These studies have indicated that phytoestrogens differ in potency among animal species. A majority of the information available on the effects of the estrogenic isoflavones has dealt with the reproductive system of sheep (Shutt and Cox, 1972; Newsome and Kitts, 1977). Little information exists on the possible adverse effects that the phytoestrogens may have in nonruminant animals. Therefore, it is difficult to make projections concerning these estrogenic effects and the potential hazard that the phytoestrogens might present to human health.

Several studies investigating the estrogenic character of the soybean isoflavones have used mice as the experimental animal. An increase in uterine weight by immature females is considered a positive response. Most reports do not indicate the strain of mice used (Cheng et al., 1963; Bickoff et al., 1962). In one report, Fredericks et al. (1981) indicate a difference in response to coumestrol, an alfalfa

phytoestrogen, between the ICR mouse and B6D2F1 mouse.

Our paper reports bioassay data showing that CD-1 mice do not respond to phytoestrogen levels comparable to those used with other strains of mice. This strain also required higher levels of diethylstilbesterol to produce an increase in uterine weight than reported previously.



## MATERIALS AND METHODS

Swiss albino CD-1 mice were obtained from Charles River Breeding Labs (Portage, Michigan). Female mice were received with the mothers at ages ten, nine, eight and seven days old. Upon arrival, mothers and pups were housed together in individual cages. Mice received food and water ad libidum. The dietary protein source was casein in a pelleted semi-purified diet obtained from United States Biochemical Corporation (Cleveland, Ohio). It was essential for the experiment to avoid exposure of the young mice to a soybean protein diet. Soybean protein-based diets have been reported to contain substantial quantities of phytoestrogens (Drane et al., 1980; Murphy et al., 1982). The composition of the semi-purified diet used is shown on table 1. Mice were weaned at 20 or 21 days old. Weanlings weighing between 8 and 11 g were randomly assigned to treatments according to a completely randomized design.

Diethylstilbesterol (DES), used as a positive control, was obtained from Sigma, lot number 49C-0092 (St. Louis, MO). Genistein (lot numbers 36471-A and 31667-A) was from ICN Rare and Fine Chemicals (Plainview, NY). Genistin and daidzin were purified from toasted soyflakes obtained from A. E. Staley Co. (Des Moines, IA) by a method developed in our lab (Murphy, 1981) and by a modification of that used by Ohta et al. (1979).

The compounds under test were suspended in 5% Tween 80 (lot number 35B-2480, Sigma Chemical Company) and were administered to the mice by stomach intubation in four daily doses of 0.1 ml per day beginning on

the first day of weaning. The control group was given 0.1 ml of 5% Tween 80 per day for the four-day period. Preliminary experiments showed no adverse or estrogenic effects due to the carrier. On the fifth day after weaning, mice were weighed and subsequently sacrificed, their uteri dissected out and weighed immediately without blotting (Bickoff et al., 1962). Total doses of the compounds administered were 0.6 and 0.8  $\mu$ g per mouse of DES, 6 and 8 mg per mouse of genistein and 12 mg per mouse each of genistin and daidzin.

Statistical analysis of the data was carried out by using a one-way classification analysis of a completely randomized design (SAS Institute Inc., Box 8000, Cary, N.C. 27511). Analysis of covariance, with uterus weight as the dependent variable of initial and final weights, was carried out to minimize any effects due to weight differences during the experiment. The ratio of uterine weight to final body weight was also calculated. This was another way to adjust for weight effects. The ratio calculated was subjected to statistical analysis. Final weight and weight gain as a function of treatments was subjected to statistical analysis to determine any effects on the final weight of the animals due to treatments.

## RESULTS AND DISCUSSION

Statistical analysis of the data revealed that the initial and final body weights were significant variables. With use of two different models incorporating initial and final weights of the animals as dependent variables, initial weight was determined to be more significant than final weight for the analysis. The animal weights were therefore adjusted with respect to initial weight only and then subjected to statistical analysis. The data are presented in table 2. Corrected mean uterine weights are the data obtained after adjustment for initial weight differences.

Statistical analysis of both corrected and uncorrected uterine weights indicated no differences between the control and the isoflavones, genistein and genistin. A significant increase in uterine weight was observed for DES and daidzin as compared with the controls.

Statistical analysis using the ratio of uterine weight to final body weight indicated no differences between the control and the isoflavones, genistein, daidzin and genistin. Statistical analysis of the data adjusted for both initial and final weight was not carried out. We feel the observed differences between the control group and the group given daidzin probably was due to final weight effects rather than an estrogenic effect because the analysis of the ratio of uterus to body weight did not show significant differences between the control and the one given daidzin.

Weight gain during the experiment between the treatments and the

control was not statistically different but was generally lower in the experimental groups than in the control. The isoflavones, genistein and genistin, were reported previously to depress body weight gain in male mice (Carter et al., 1960). Final weight, although not statistically different between treatments, appeared to be an important variable in this experiment. Further experimentation is required to enable us to draw conclusions on how final weight and weight gain are affected by treatments in this particular type of experiment.

The doses of genistein used in this experiment have been reported by other researchers to increase uterine weight in mice of unknown strain (Bickoff et al., 1962). The doses of DES used in this experiment were ten times higher than those reported by Bickoff et al. (1962) to cause increased uterine weights. Doses of DES fed at levels comparable to Bickoff et al. (1962) gave no response in the CD-1 mouse (data not shown). The CD-1 mice have been used by other researchers (Leavitt and Wright, 1963; Leavitt and Wright, 1965) in similar experiments to measure estrogenic potency. These experiments were for a duration of ten days. The compound under test was coumesterol. A total dose of 400  $\mu$ g coumesterol per mouse was required to produce an increase in uterine weight of 4 mg over the control. The same total dose of coumesterol was reported by Bickoff et al. (1962) to increase uterine weight by 30 mg over the control in mice of an unknown strain.

The results of our experiment combined with the previous reports (Leavitt and Wright, 1963, 1965) indicate that the CD-1 mouse does not respond to doses of estrogenic compounds that have produced responses in

other strains of mice. Therefore, comparisons made between experiments using this strain of mouse with experiments using other strains should be cautiously interpreted. The reported differences in response among strains of mice indicates that a standardization of the estrogen bioassay with respect to the strain of mouse is required.

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Table 1. Composition of semi-purified diet<sup>a</sup>

Component	Percent (by weight)
Casein High Nitrogen	20.0
DL-Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiber-Celufil	5.0
Corn oil	5.0
AIN Mineral mix	3.5
AIN Vitamin mix	1.0
Choline bitartrate	0.2

<sup>a</sup>Source: United States Biochemical Corporation, AIN Rat and Mouse Diet 76.



Table 2. Body-weight and uterine-weight response data for weaning CD-1 mice treated with diethylstilbestrol, genistein, genistin or daidzin by gastric intubation for 4 days

Data	Dose	Values <sup>a</sup> for		
		5% Tween 80 <sup>b</sup>		Genistein
		0.1 ml	6 mg	8 mg
No. of mice/group		56	16	13
Mean body weight (g):				
Initial		9.6	9.4	9.4
Gain (5 days)		3.5	3.4	3.0
Final		13.1	12.7	12.4
Uterine weight (mg):				
Mean		25.4	23.8	22.0
Corrected mean		25.1	25.3	23.1
Uterine weight/body				
weight ratio x 1000		1.93	1.87	1.77

<sup>a</sup>Values are means for the numbers of mice stated.

<sup>b</sup>Vehicle control.

<sup>c</sup>Positive control.

<sup>d</sup>Denotes a significant difference from the control value at  $P < 0.05$ .

mice treated with				
DES <sup>c</sup>		Genistin	Daidzin	
0.6 $\mu$ g	0.8 $\mu$ g	12 mg	12 mg	
15	11	8	8	
9.6	10.0	9.9	10.5	
2.1	2.1	3.6	3.7	
11.7	12.1	13.5	14.2	
77.7 <sup>d</sup>	79.5 <sup>d</sup>	24.0	27.0	
77.9 <sup>d</sup>	77.0 <sup>d</sup>	25.1	28.9 <sup>d</sup>	
6.61	6.57	1.77	1.90	

## ACKNOWLEDGEMENTS

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PART III. ESTROGENIC RESPONSE OF ICR B6D2F1 AND B6C3F1 STRAINS OF MICE  
GIVEN DES ORALLY

by

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Running Title: Estrogenic Response to Diethylstilbestrol.

Submitted to: Food and Chemical Toxicology

## ABSTRACT

The estrogenic response of three strains of mice, ICR, B6D2F1 and B6C3F1, was tested with respect to diethylstilbestrol. The B6D2F1 and B6C3F1 strains were found to be two times more sensitive to diethylstilbestrol than was the ICR mouse.

## INTRODUCTION

A recent report from this laboratory (Farmakalidis and Murphy, 1984) described the estrogenic response of the CD-1 mouse to the soybean isoflavones genistein, genistin and daidzin. The mice did not respond to levels of genistein and diethylstilbestrol (DES) previously reported to cause an increase in uterine weights in immature female mice (Bickoff et al., 1962). An increase in uterine weights by immature female mice is considered a positive response to estrogenic compounds. The amount of DES required to cause an increase in uterine weight was greater. Most reports investigating the estrogenic character of the isoflavones with use of mice as the experimental animal do not report the strain of mouse used. One report, Fredericks et al. (1981) indicated a difference in response to coumesterol, an alfalfa phytoestrogen, between the ICR mouse and the B6D2F1 mouse.

With the apparent difference in response with respect to the strain of mouse used in such experiments, we decided to investigate the estrogenic response of different strains of mice in an effort to determine which strain would be best suited for later experiments with the soybean isoflavones. DES was the compound used (the positive control in later experiments).

This paper presents data showing the estrogenic response of ICR, B6D2F1 and B6C3F1 strains to DES at a level used previously by Bickoff et al. (1962).

## MATERIALS AND METHODS

Outbred white albino ICR and inbred B6D2F1 and B6C3F1 mice were obtained from Harlan Sprague Dawley (Madison, Wisconsin). Female mice at the age of 12 days were received with the mothers. Upon arrival, mothers and pups were housed together in individual cages. Mice received food and water ad libitum. The diet (United States Biochemical Corporation, Cleveland, Ohio) was the same as the one used previously (Farmakalidis and Murphy, 1984). Mice were weaned at 20 or 21 days old. Weanlings weighing between 12 and 15 g for the ICR strain and between 7 and 9 g for B6D2F1 and B6C3F1 were randomly assigned to treatments according to a completely randomized design.

Diethylstilbestrol (DES), used as the estrogen to determine estrogenic response and to be used as a positive control in later experiments, was obtained from Sigma, lot number 49C-0092 (St. Louis, Missouri).

DES was suspended in 5% Tween 80 (lot number 23F-0030, Sigma Chemical Company) and was administered to the mice by stomach intubation in four daily doses of 0.1 ml per day beginning on the first day of weaning. The control group was given 0.1 ml of 5% Tween 80 per day for the four-day period. On the fifth day after weaning, mice were weighed and subsequently sacrificed, their uteri dissected out and weighed immediately without blotting (Bickoff et al., 1962). The total dose of DES administered was 0.1 µg/mouse.

Statistical analysis of the data was carried out using a one-way

classification analysis of a completely randomized design (SAS Institute Inc., Box 8000, Cary, N.C. 27511). Analysis of covariance, with uterus and final weights as the dependent variables of initial weight and treatments, was carried out to minimize any effects due to weight differences between the animals during the experiment. The ratio of uterine weight to final body weight also was calculated. This was another way to adjust for weight effects. The ratio calculated was subjected to statistical analysis. Final weight and weight gain as a function of treatment was subjected to statistical analysis to determine any effects on the final weight of the animals due to treatments.



## RESULTS AND DISCUSSION

Statistical analysis of the data revealed that the initial and final weights of the animals within a particular strain of mouse were not significant variables affecting the weight of the uterus. Statistical analysis of the uterine weights indicated a difference between the control and experimental group for all three strains of mice used. The experimental groups of the inbred strains of mice had a greater increase in uterine weights over the controls with the same dose of DES than did the ICR (outbred) strain. The results are shown on Table 1. There was no significant difference in the increase of uterine weights between the two inbred strains of mice. Variation between animals within a strain was small for the B6D2F1 and B6C3F1, whereas variation within the ICR strain was larger. The variation in response between animals observed was expected because the ICR strain, being outbred, tends to be more heterogeneous, whereas the other two strains tend to be more homogeneous (Festing, 1979, pp. 54-63; Russell and Burch, 1956, pp. 105-109).

Comparisons made using the ratio of uterine weights over final weights indicated no significant differences between the three different strains of mice in respect to DES. However, there was a significant difference in the increase of the ratio of the experimental group over the control between the ICR strain and the two inbred strains.

The increases in uterine weights obtained in this experiment with strains B6D2F1 and B6C3F1 mice are comparable to the increase in uterine

weights obtained by Bickoff et al. (1962) in his experiment for the same dose of DES. In contrast, our previous results with the CD-1 mouse (Farmakalidis and Murphy, 1934) did not respond to the dose of DES used in this experiment. A 1- $\mu$ g/mouse dose caused a threefold increase in uterine weight in the CD-1 mouse.

The results of this experiment, combined with our previous report, indicate that differences do exist among strains of mice with respect to their response to estrogen. Outbred mice are larger animals. Therefore, the absolute dose of an estrogenic compound per gram weight is less than the absolute dose per gram weight for the inbred strains. Whether the difference observed is due to this weight differential or reflects genetic differences cannot be determined at this point. The results obtained in this experiment show that the two inbred strains of mice are more sensitive to DES than is the ICR strain. This report does not offer a standardized method for testing estrogenic compounds. However, it does provide more information regarding the differences in response that exist between different strains of mice.

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Table 1. Body uterine weights<sup>a</sup> of three strains of mice given 0.1 µg diethylstilbestrol (DES) orally

Group	No. mice	DES dose (µg/g initial body wt)	Initial body wt (g)
ICR			
Control	12	-	13.45
DES	12	0.0075	13.37
B6D2F1			
Control	12	-	8.01
DES	12	0.0125	8.01
B6C3F1			
Control	16	-	8.90
DES	16	0.0115	8.66

<sup>a</sup>Weights are means for the number of mice indicated.

<sup>b</sup>Values are significantly different from the corresponding control value at  $P < 0.05$ .

<sup>c</sup>Value is significantly different from each of the corresponding values from the other two strains.

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Final body wt (g)	Uterine wt (mg)	Difference in uterine wt (mg)	Ratio of uterine wt (mg)/final body wt (g)	Difference in ratios
17.58	27.30		1.55	
16.59	29.90	2.60	1.80 <sup>b</sup>	0.25 <sup>c</sup>
10.90	12.66		1.16	
11.47	24.34	11.68	2.12 <sup>b</sup>	0.96
11.16	13.70		1.23	
11.16	26.00	12.90	2.33 <sup>b</sup>	1.10

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PART IV. ESTROGENIC POTENCY OF GENISTIN AND DAIDZIN IN MICE

by

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## ABSTRACT

The estrogenic activity of genistin and daidzin was investigated by using the B6D2F1 strain of mouse. Diethylstilbestrol and genistein were used as positive controls. The estrogenic response to 1.5 mg of genistin was equivalent to 1 mg of genistein, giving a 1:1 molar relationship in estrogenic activity between genistin and genistein. The estrogenic response to 3.8 mg of daidzin was equivalent to 1 mg of genistein. Analysis of the blood obtained from the mice in this experiment failed to reveal any free genistein or daidzein in plasma.



## INTRODUCTION

The possible significance of naturally occurring estrogens in foods has been widely publicized by the debate over the presence of diethylstilbestrol (DES) residues in liver or other tissues of animals treated with DES as a growth stimulant. DES was recently banned by the FDA (Grunby, 1980). Amounts of DES present in meat and poultry products that led to the ban were very small. It has been frequently suggested that the dietary estrogens to which humans are exposed are predominantly naturally occurring phytoestrogens rather than residues of feed additives. Several reports (Knuckles et al., 1976; Lookhart, 1980; Young et al., 1977; Verdeal and Ryan, 1979) have indicated that some common foods contain levels of phytoestrogens that are 3-4 times higher than an equivalent dose of 0.5 ppb DES, a large overestimate of actual DES levels in 100 g of mishandled beef liver (CAST, 1977). Recent reports have indicated that the concentrations of soy phytoestrogens calculated to match 0.5 ppb DES was found to be well within the concentration range of most soy products examined (Murphy, 1982). It seems probable then, that actual human exposure to phytoestrogens is substantially higher than the potential human exposure to DES residues (Verdeal and Ryan, 1979).

The isoflavones, genistein and daidzein, isolated from strains of subterranean clover, were identified as the active estrogenic compounds responsible for interference in the estral cycle of sheep, causing a serious reduction in lambing percentages (Bennets et al., 1946). These

compounds also were isolated from soybeans nearly 50 years ago (Walz, 1931). Genistein and daidzein are roughly  $10^5$  times less effective estrogens than DES in mice as shown by uterine enlargement assays (Bickoff et al., 1962; Millington et al., 1964). Bickoff et al. (1962) reported that 8 mg of genistein, 11 mg of daidzein and 0.083  $\mu$ g of DES were required to produce a 25 mg uterus in mice. The strain of mouse used in the experiment was not reported.

Animal species differ in their response to phytoestrogens. In sheep, the estrogenic activity of different varieties of clover was correlated with the formononetin content, but not with the content of genistein or biochanin A (Braden et al., 1967). In contrast, formononetin was found to be much less active than genistein when fed to mice (Bickoff et al., 1962). Sensitivity to phytoestrogens also was found to differ between animal species. Sheep are more sensitive than mice (Morley et al., 1968). Recent reports from this laboratory (Farmakalidis and Murphy, 1984a, b) indicate a difference between strains of mice with respect to estrogenic response to DES and the soybean isoflavones genistein, genistin and daidzin. The Swiss albino CD-1 mouse did not respond and the ICR mouse had a slight response to estrogen levels reported earlier to cause uterine enlargement in mice (Bickoff et al., 1962). The B6D2F1 and B6C3F1 strains had estrogenic responses similar to those reported by Bickoff et al. (1962) for the same levels of estrogen.

It was suggested (Newsome and Kitts, 1977) that the phytoestrogen concentrations in the blood of farm animals consuming estrogenic forages

could be high enough to compete with endogenous estrogens for sites on the estrogen-binding protein. Concentrations of phytoestrogens could be well over 100 times more than the concentration of circulating 17- $\beta$ -estradiol (Newsome and Kitts, 1980). For example, Shemesh et al. (1972) measured a plasma coumestrol level of 3.9 ng/ml in goats consuming alfalfa containing 24 ppm coumestrol. This plasma level is 975 times the circulating level of 4 pg 17- $\beta$ -estradiol/ml as reported by Scaramuzzi and Land (1978). These low levels of phytoestrogens have been related to reproductive disorders in animals through their ability to suppress estrus.

The use of soy protein in foods for human use is increasing. The isoflavonoids, genistin and daidzin, constitute 90-95% of the isoflavone content in soybeans. Recent reports (Murphy, 1982; Eldridge, 1982) indicate a significant carry-over of these forms to processed soybean products. The estrogenic potency of daidzin and genistin has not been investigated.

Our paper reports data on the estrogenic potency of genistin and daidzin and their possible significance as estrogenic compounds finding their way into human foods. Blood plasma was assayed for free genistein and daidzein.

## MATERIALS AND METHODS

Inbred B6D2F1 mice were obtained from Harlan Sprague Dawley (Madison, Wisconsin). The experimental procedure used was the same as reported previously (Farmakalidis and Murphy, 1984a). Total doses of the compounds per mouse administered were: 0.12 µg of DES, 8 mg of genistein and 12 mg each of genistin and daidzin. Animals were assigned to treatments according to a completely randomized design.

Diethylstilbestrol (DES) used as a positive control was obtained from Sigma Chemical Co. (St. Louis, MO; lot no 49C-0092). Genistein was obtained from ICN Rare and Fine Chemicals (Plainview, N.Y.; lot nos 36471-A and 31667-A). Genistin and daidzin were purified from toasted soyflakes, obtained from A. E. Staley Co. (Des Moines, IA), by a combination of methods. Briefly, soyflakes were extracted overnight with acetone and 0.1N HCl in the ratio of 5:1 (ml/g of flakes) (Murphy, 1981). After filtration through Whatman filter number 1 the extract was concentrated by using a rotary evaporator at 40°C, and the extract was passed through a 2.5x50 cm silica gel column (60-80 mesh, Baker Chem. Co. Jackson, TN) equilibrated with chloroform:methanol (9:1). The isoflavones genistin and daidzin were eluted from the column with 250 ml of chloroform:methanol (50:50). The solvent was evaporated, and the residue was redissolved in the minimum amount of methanol. Three volumes of water were added, and the solution was left to stand in the refrigerator for two days. The precipitate was dissolved in 100% ethanol and chromatographed through a 2.5x50 cm Sephadex LH-20 column

equilibrated with 100% ethanol (Ohta et al., 1979). The mixture was fractionated into genistin and daidzin by using 100% ethanol. Genistin (m.p.  $256\pm 2^{\circ}\text{C}$ ) was obtained in pure form by repeated precipitation from a supersaturated solution of 100% ethanol. Daidzin (m.p.  $230\pm 2^{\circ}\text{C}$ ) was crystallized twice from 25% methanol and once from 50% methanol. The identity and purity of the compounds obtained by this procedure was confirmed by using nuclear magnetic resonance spectroscopy, melting points, ultraviolet and infrared spectroscopy, thin-layer and high-performance liquid chromatography (HPLC), and through acid and enzymatic hydrolysis (Mabry et al., 1970, p. 71).

Analysis of blood plasma was carried out by using reverse-phase high-performance liquid chromatography (HPLC) utilizing a gradient of methanol-water (Murphy, 1981). A method for extracting genistein and daidzein from blood plasma was developed. Briefly, blood obtained from mice in each experimental group was combined. Heparin was used as an anticoagulant. Shortly after collection blood was centrifuged at  $4^{\circ}\text{C}$  at  $5,000g$  for 20 minutes to precipitate cells. Two volumes of acetonitrile was added to the plasma, and the mixture was centrifuged at  $10^{\circ}\text{C}$  at  $10,000g$  for 30 minutes. The supernatant was further purified through a silica gel Waters Sep-Pak cartridge (Millipore Inc. Milford, Mass.) by using 2 ml of 33% acetonitrile. After filtration through a  $0.45\ \mu\text{m}$  filter, the extract was taken to dryness, redissolved in  $20\ \mu\text{l}$  methanol and injected on the HPLC through a  $20\ \mu\text{l}$  loop. Earlier experiments indicated a 95% recovery of standards added to the plasma before extraction. The lowest detectable level of genistein and

daidzein, by using ultraviolet absorption at 254nm, was 0.5 µg/ml.

Statistical analysis of the data was carried out by using a one-way classification analysis of a completely randomized design, with the uterine weight being the only dependent variable on the treatments. Previous experiments (Farmakalidis and Murphy, 1984b) indicated that initial and final weights of the animals for this strain of mouse were not important variables to be included in the analysis. Comparisons between the mean uterine weights was carried out by using the Students t-test (SAS Institute Inc., Box 8000, Cary N.C. 27511).

## RESULTS

There were significant ( $P < 0.05$ ) differences in estrogenic potency of soybean isoflavone glucosides between the control and all the experimental groups, except for the one given daidzin (Table 1). Relative potencies, defined as the ratio of the doses calculated for the various compounds required to produce an increase in uterine weight equivalent to 8 mg of genistein, are given in table 1. There was no significant difference between the group given 8 mg of genistein and the one given 12 mg of genistin. This indicates a 1:1 molar estrogenic potency relationship between the two compounds. The dose of genistein used for this experiment was reported earlier to produce a 25 mg uterus in mice (Bickoff et al., 1962). The 1:1 molar relationship between the compounds would suggest that either genistin is completely hydrolyzed in the gut to yield genistein or that the absorption of the two compounds is the same. Because the metabolism of isoflavones differs in animal species (Braden et al., 1967; Morley et al., 1968), the possibility that genistin is metabolized to a compound other than genistein that exhibits an estrogenic response equivalent to genistein is not excluded by these data.

The results for daidzin at the dose used in this experiment are inconclusive. Although mean uterine weights were greater than those of the control, they were not statistically significant ( $P = 0.2$ ). In comparison with the dose of genistein used in this experiment, 3.8 mg of daidzin produced a uterine enlargement equivalent to 1 mg of genistein.

Daidzein was reported to be less effective as an estrogen than genistein when fed to mice (Bickoff et al., 1962). Eleven milligrams of daidzein were required to produce a 25 mg uterus in mice (Bickoff et al., 1962). The relative potency of daidzein was reported to be 0.75 (Bickoff et al., 1962). This value is much higher than the relative potency of daidzin calculated in this experiment. This suggests to us that daidzin is less effective as an estrogen than daidzein on a molar basis. Thus, it was not surprising that the dose of daidzin used in this experiment did not elicit a high estrogenic response. Daidzein was not used as a positive control in this experiment. Thus, a direct comparison between daidzin and daidzein cannot be made. Genistin was less effective as an estrogen than genistein as shown by the relative potency values calculated (Table 1). The relative potencies for DES and genistein calculated in this experiment were comparable to those reported earlier (Bickoff et al., 1962). The increase in uterine weights, however, was not the same for an equivalent dose of estrogen. This difference was attributed to the fact that there are differences in response to estrogens between strains of mice (Farmakalidis and Murphy, 1984a, b). It is evident from the results that daidzin is much less effective as an estrogen than is genistin. However, both genistin and daidzin can increase uterine weight.

The concentration of phytoestrogens circulating in the plasma would reflect the amount of hormonally active substance likely to reach target tissues. With our method, the lowest detectable limit for genistein and daidzein was 0.5 ng/ $\mu$ l of standard solutions of genistein and daidzein.



Since the total plasma volume obtained for analysis was 1.2 ml for genistin and 0.8 ml for daidzin the lowest detectable concentrations of free isoflavones would have to be 13.1 ng/ml plasma for daidzein and 3.7 ng/ml plasma for genistein. (Total plasma obtained was extracted and concentrated to an HPLC injection volume of 20  $\mu$ l.) Free genistein and daidzein could not be detected in the plasma at the levels that could be analyzed with our HPLC system. Analysis for free genistein and daidzein by high-performance liquid chromatography at levels lower than those that could be detected in this system would require the use of a more sensitive detector. Alternatively, a more concentrated sample could be used, which implies that greater numbers of mice than were used in these experiments are required. It is possible that genistein and daidzein may be present in the plasma bound to glucuronic acid rather than as free forms. However, we have not analyzed the plasma for these forms. Another possible explanation for the results would be that genistin and daidzin are not metabolized to the aglucones, but to some other compounds that are also estrogenic. This has been reported in other species (Shutt *et al.*, 1970; Batterham *et al.*, 1971).

## DISCUSSION

The adverse effect of the isoflavones in animal reproduction is well known (Moule et al., 1963; Morley et al., 1968). However, most of the information available is mainly concerned with the isoflavones, genistein and daidzein. Our results have shown that daidzin and genistin are also estrogenic. Therefore, their presence in human foods should not be ignored.

It would be of interest to calculate an approximate amount of isoflavones that would produce an estrogenic effect in humans. The amount was estimated on the basis of food intake per day of mice and humans. The compounds were administered to the mice by stomach intubation; thus, we can express the dose given as ppm in the diet. The mice used in this experiment consumed, on the average, 2 g food/day. Since the average weight of a mouse was 10 g the consumption of food represents 20% of their body weight per day. Mice were administered 3 mg of the compounds per day, which represents a dose of 1500 ppm in the diet. Humans consume about 1.5 kg of food per 60 kg body weight per day. The consumption of food represents 2.5% of the body weight. Using the relation  $20/2.5 \times (y \text{ ppm in diet of mouse}) = z \text{ ppm in diet of humans}$ , the amount calculated is approximately 8,000 ppm in the diet/day, present as genistin. The amount of genistin present in a number of processed soybean products was found to range between 37 ppm in breakfast patties to 1,601 ppm in toasted defatted flakes (Murphy, 1982). The potential human exposure to phytoestrogen concentration

required for a physiological response, thus seems to be well within reach. However, soybean products commonly used for human consumption contain much lower concentrations of phytoestrogens than those found in toasted defatted soyflakes (Murphy, 1982; Eldridge, 1982). We can assume then, that the levels of soy phytoestrogens to which humans are likely to be exposed are lower than those required for an estrogenic response. It is possible that humans are more sensitive to phytoestrogens than mice. The low-dose long-term effect resulting from exposure to phytoestrogens should not be ignored especially, because amounts lower than those required for uterine enlargement effects have also been reported to cause adverse effects in mice (Fredericks et al., 1981; Folman and Pope, 1966).

The phytoestrogens coumestrol and genistein are thought to affect fertility in mice by inhibiting release of follicle-stimulating hormone (FSH) (Smith et al., 1979). Inadequate levels of FSH in the final stages of follicle development may decrease ovulation rate and delay ovulation, thereby increasing the incidence of degenerate embryos (Butcher et al., 1974; Hirshfield and Midgley, 1974). Fertility in mice was found to be significantly lowered with a dietary coumestrol level of 50 ppb (Fredericks et al., 1981). This level is much lower than the 20 ppm of dietary coumestrol required for uterine enlargement effect (Bickoff et al., 1962). The phytoestrogens also have been reported to act as antiestrogens. Estrogenic compounds can be agonistic or antagonistic to 17- $\beta$ -estradiol when they both act on the target tissue simultaneously (Clark et al., 1977). Antagonistic compounds generally

compete for 17- $\beta$ -estradiol receptors and fail to stimulate the nucleus to respond fully (Anderson et al., 1972). Folman and Pope (1966), using the uterine weight bioassay, found that high doses coumestrol and genistein were antagonistic to 17- $\beta$ -estradiol. The doses of coumestrol and genistein required for this effect were still lower than the dose required to cause an increase in uterine weights (Bickoff et al., 1962).

The sources of phytoestrogens in the human diet are fresh fruit, such as apples and cherries, vegetables (e.g., potatoes), soy products, condiments such as garlic, hops used in beer production and other processed plant products (Miller, 1977). Epidemiological studies have shown a greater prevalence of breast cancer in women living in countries consuming a diet rich in fat (Western diet) than in those living in countries consuming a vegetarian or semivegetarian diet (Miller, 1977; Wynder, 1980). Phytoestrogens have been shown to bind with relatively high affinities to the estrogen receptors of human mammary tumor cells (Martin et al., 1978; Verdeal et al., 1980). In view of the evidence that the compounds may act as antiestrogens (Tang and Adams, 1980), their presence in substantial concentrations in the vegetarian diet may offer an explanation for this difference.

Recent studies (Bannwart et al., 1984) have drawn attention to the fact that the isoflavones daidzein and equol have been detected in human urine from vegetarian women. The concentrations of daidzein detected ranged from 0.4  $\mu\text{mol/l}$  to 4.6  $\mu\text{mol/l}$ , with corresponding equol concentrations ranging from 6.17 to 39.93  $\mu\text{mol/l}$ . In sheep, the metabolic products of formononetin were reported to be daidzein and

equol (Shutt et al., 1970; Batterham et al., 1971). Equol was found to be a more potent estrogen than daidzein in sheep. It might be possible that daidzein is partly metabolized to equol in the human body, following a metabolic pathway similar to the one in sheep. The importance of equol production in the human body probably would have a different implication than it has in sheep because equol was found to be less potent as an estrogen than daidzein in mice. Equol has a low binding affinity to sheep and rat uterine cytosol estrogen receptors and has a uterotrophic potency of  $1 \times 10^{-3}$  that of 17- $\beta$ -estradiol in immature rats (Tang and Adams, 1980; Shutt and Cox, 1972). Equol can act as an antiestrogen at doses that elicit a weak uterotrophic response (Tang and Adams, 1980). The importance of equol production in the human body would be its effect as an antiestrogenic compound rather than as an estrogen. The metabolic fate of the isoflavones in humans and the effect that the metabolites might have still remain to be investigated.

The presence of phytoestrogens in the human diet requires careful consideration. Genistin and daidzin exhibit an estrogenic response in mice at high doses. However, the importance of phytoestrogens in the diet may lie as much in their ability to exert adverse or beneficial effects at low doses as in their own estrogenic activity at higher doses. Their effects on human reproduction or hormone-dependent tumors have not been studied. Further studies are required to clarify whether these compounds have any important biological role in man.

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Table 1. Estrogenic potency of soybean isoflavone glucosides

Treatment	number of animals	mean uterine weight (mg)	Relative potency <sup>a</sup>
Control	24	14.88 ± 0.91	
DES	24	26.60 ± 0.91 <sup>b</sup>	100,000
Daidzin	16	17.32 ± 1.28 <sup>c</sup>	0.26
Genistin	26	20.47 ± 0.90 <sup>b,c</sup>	0.56
Genistein	7	21.07 ± 1.87 <sup>b,d</sup>	1.00

<sup>a</sup>Genistein arbitrarily assigned a value of 1.00.

<sup>b</sup>Means are significantly different from control at P<0.05.

<sup>c</sup>Mean is significantly different from control at P=0.2.

<sup>d</sup>Means are not significantly different from each other.

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PART V. ISOLATION OF 6"-O-ACETYLGENISTIN AND 6"-O-ACETYLDAIDZIN FROM  
TOASTED DEFATTED SOYFLAKES

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## ABSTRACT

The isoflavones, 6"-0-acetylgenistin and 6"-0-acetyldaidzin, were isolated and purified from toasted defatted soyflakes. Their structure was confirmed with nuclear magnetic resonance spectroscopy, mass spectroscopy, ultraviolet and infrared spectroscopy, and acidic and enzymatic hydrolysis. Defatted soyflakes contained 52 ppm of 6"-0-acetyldaidzin and 38 ppm of 6"-0-acetylgenistin. Acetone with 0.1N HCl was found superior to 80% methanol for extraction of these compounds.

## INTRODUCTION

Soybeans contain the estrogenic isoflavones genistein, daidzein, and their glucosides, genistin and daidzin (Naim et al., 1974; Eldridge, 1982a; Murphy, 1981). Gyorgy et al. (1964) reported the presence of 6,7,4'-trihydroxyisoflavone in fermented soybeans (tempeh), which was a product of the fermentation process. Naim et al. (1973) reported the presence of the isoflavones, glycitin, and glycitein. The compounds are the 6-methoxy derivatives of daidzin and daidzein, respectively. Eldridge (1982a, b, 1983) also reported the presence of glycitin and glycitein in a variety of soybean products analyzed by high-performance liquid chromatography. Other researchers (Ohta et al., 1979, 1980) reported that glycitin and glycitein were not detected in their extracts. Instead 6"-0-acetylgenistin and 6"-0-acetyldaidzin were detected. The structures of the isoflavone glucosides found in soybeans are shown in figure 1. Earlier reports from our laboratory (Murphy, 1982) have drawn attention to two additional unidentified peaks present in chromatograms of several soy-protein-based foods analyzed by high-performance liquid chromatography.

To date, there has been only one report (Eldridge, 1982b) that gives quantitative data on glycitin and glycitein in soybeans and soy-protein products. No quantitative data exist for 6"-0-acetylgenistin and 6"-0-acetyldaidzin. Bioactivities for glycitein, glycitin, and the two acetylisoflavones as estrogens have not been reported.

In view of the differences observed between researchers concerning

the natural occurrence of soy isoflavones, it was of interest to isolate and characterize the compounds contained in peaks 3 and 4 (figure 2) observed in our chromatograms. Two solvent systems were compared for extraction efficiency of the two isoflavones isolated from soyflakes.

## MATERIALS AND METHODS

## Extraction and Isolation

Toasted defatted soyflakes were obtained from A. E. Staley and Co. (Des Moines, IA). Two kilograms of soyflakes were extracted by stirring with acetone and 0.1N HCl overnight in the ratio of 5:1 (ml/g flakes). After filtration through Whatman filter paper number 1, the extract was concentrated on a steam bath, then dried by using a rotary evaporator at 40°C. The crude extract was suspended in chloroform:methanol (9:1) and chromatographed through a 4 x 50 cm silica gel column (60-80 mesh, J. T. Baker, Knoxville, TN). The column was washed with 400 ml of chloroform:methanol (9:1). The compounds under investigation were eluted from the column with 250 ml of chloroform:methanol (85:15). After evaporation of the solvent, the residue was dissolved in 50% water-saturated ethyl acetate and chromatographed on a 2.5 x 50 cm silica gel column (50-80 mesh) according to the method of Ohta et al. (1979). Partial separation of the two new isoflavones was achieved at this point. Further fractionation to the individual compounds was achieved by chromatography on a 2.5 x 50 cm Sephadex LH-20 column with 100% ethanol (Ohta et al., 1979). Purification of the compound contained in peak 3 was necessary at this point to remove impurities, mainly daidzin. This was accomplished by repeated chromatography through a silica gel column equilibrated with 50% water-saturated ethyl acetate (Ohta et al., 1979). The compound in peak 3 (6"-0-



acetylaidzin) was precipitated twice from 90% methanol. The compound in peak 4 (6"-O-acetylgenistin) was precipitated twice from 25% methanol and once from 20% ethanol.

Ultraviolet (uv) spectra (in methanol) were measured in a Beckman DK-2 spectrophotometer. The effects of  $AlCl_3:HCl$ , sodium methoxide, and sodium acetate on the uv absorption maxima were carried out according to Mabry et al. (1970, pp. 36-37). Infrared spectra (on KBr disc) were performed in a Beckman IR-2 spectrophotometer. The proton magnetic resonance (pmr) spectrum of the tetramethylsilated derivative (Mabry et al., 1970) of 6"-O-acetylgenistin was performed on a Bruker WM-300 spectrophotometer with tetramethylsilane (TMS) as an internal standard. Electron impact mass spectra (ms) were recorded on a Finnigan model 3680A and AE1 MS902 for exact mass. Chemical ionization mass spectrometry with isooctane was performed on a Finnigan model 6817A. Analytical thin-layer chromatography (TLC) was performed on silica gel G (Fisher Scientific Co.), with the solvent systems: A. chloroform:methanol (9:1) (Beck, 1964); B. benzene:ethyl acetate:petroleum ether (b.p. 40-60°C):methanol (6:4:3:1) (Barz, 1969); C. ether:petroleum ether (b.p. 40-60°C) 7:3 (Barz, 1969) D. chloroform:methanol:water (65:25:4) (Wang, 1971). Spots were visualized with the aid of a uv lamp at 366nm (Beck, 1964). Enzymatic hydrolysis with almond  $\beta$ -glucosidase (Sigma Chemical Co., St. Louis, MO) and acidic hydrolysis with 6% HCl was carried out according to Mabry et al. (1970, pp. 24-25). Elemental analysis was performed by Gailbraith Laboratories (Knoxville, TN). Analysis of the carbohydrate moiety was performed by

analytical thin-layer chromatography on Kieseguhr G (Merck Co) impregnated with phosphate buffer, pH 5 (Lewis and Smith, 1969, p. 303). The solvent system used was butanol:acetone:sodium phosphate buffer, pH 5 (40:50:10) (Waldi, 1965).

#### Comparison of Extraction Methods

Samples from the same batch of toasted defatted soyflakes were extracted with (a) acetone and 0.1N HCl (Murphy, 1981) and (b) with 80% methanol (Eldridge, 1982b). The extracts obtained were analyzed by high-performance liquid chromatography (HPLC) according to Murphy (1981, 1982).

#### Identification of Compounds

Purity of the compounds was determined by melting points, thin-layer chromatography, and high-performance liquid chromatography. Melting points were not corrected.

## RESULTS AND DISCUSSION

## Identification of Compounds

The purity of the compounds isolated was confirmed by TLC, HPLC, and melting points ( $186\pm 2^\circ\text{C}$ , 6"-O-acetyldaidzin;  $196\pm 2^\circ\text{C}$ , 6"-O-acetylgenistin). TLC  $R_f$  values of the compounds in the various solvents appear in Table 1. The compounds were less polar than the glucosides, genistin, and daidzin, but more polar than genistein and daidzein as shown by TLC and HPLC analysis. This indicated that the two new compounds had fewer hydroxyl groups than the glucosides (genistin and daidzin) but more hydroxyl groups than the aglucones (genistein and daidzein).

Ultraviolet absorption maxima of 6"-O-acetylgenistin and 6"-O-acetyldaidzin in methanol and with the various reagents appear in table 2. Addition of Na-methoxide caused a bathochromic shift in the spectrum of 6"-O-acetylgenistin, indicating the presence of a hydroxyl group in the A ring. Addition of  $\text{AlCl}_3:\text{HCl}$  indicated the presence of a 5-hydroxyl group in 6"-O-acetylgenistin. The uv absorption maxima of both compounds did not show any change with the addition of Na-acetate, indicating a bound 7-OH group.

The structures of 6"-O-acetyldaidzin and 6"-O-acetylgenistin were assigned to the compounds corresponding to peaks 3 and 4 (figure 2), respectively, on the basis of the following experimental results.

Enzymatic hydrolysis of the compounds with  $\beta$ -glucosidase was not

complete and yielded a mixture of the original isoflavonoid and (a) genistein when 6"-O-acetylgenistin was used or (b) daidzein when 6"-O-acetyldaizidin was used. In contrast, acid hydrolysis yielded mixtures of genistein or daidzein with genistin or daidzin, rather than the original isoflavonoid, respectively. These results, combined with those obtained by uv spectroscopy (table 2), indicated that the compounds were derivatives of genistin and daidzin. Analysis of the carbohydrate moiety (table 3) obtained by enzymatic hydrolysis yielded a sugar that did not correspond to any of the standard sugars used. Analysis of the carbohydrate moiety obtained through acid hydrolysis revealed the presence of D-glucose. The difference in the identity of the carbohydrate moiety observed between the enzymatic and acidic hydrolysis suggested that the sugar moiety attached to the isoflavonoid nucleus was modified in some way. Enzymatic hydrolysis of the bond linking the carbohydrate moiety to the isoflavonoid nucleus would yield genistein or daidzein from the compounds and a modified sugar molecule. Acid hydrolysis produces genistin or daidzin and glucose.

The infrared spectra of 6"-O-acetylgenistin (figure, 3A) and 6"-O-acetyldaizidin (figure, 3B) showed absorption at  $1735\text{ cm}^{-1}$  that was absent from the spectrum of genistin (figure, 3C) and daidzin (spectrum not shown), respectively. The peak at  $1735\text{ cm}^{-1}$  was assigned to an aliphatic acetoxy group (Harborne et al., 1975, p. 71). Infrared peaks were assigned as follows:  $\nu_{\text{max}}$   $3400$  (OH);  $1735$  ( $\text{CH}_3\text{-CO-OR}$ );  $1625$  (C=O);  $1605$ ,  $1570$ ,  $1515$  (C=C);  $1260$  (aromatic C-O).

The pmr spectrum of 6"-O-acetylgenistin (60 MHz in  $\text{CCl}_4$ ) showed

the signal of a doublet at  $\delta=5.1$ . This was assigned to the anomeric proton of the glucose molecule, indicating a  $\beta$ -configuration of the glucosidic linkage (Mabry et al., 1970, p. 268). The signals of the sugar protons between 3.6 and 3.9 were assigned to the protons at 2"-, 3"-, and 4"- of the glucose molecule. The signals between 4.1 and 4.5 were assigned to 5"- and 6"- protons, seemingly because of the downfield shifts due to the acetyl group (Higuchi and Donnelly, 1973). The signals for the aromatic protons were observed at  $\delta=7.4$  (2'-H, 6'-H) 6.69-6.95 (3'-H, 5'-H, 8'-H) 6.4 (6-H) 2.1 (acetyl group), 7.8 (2-H).

The molecular formula  $C_{23}H_{22}O_{11}$  was assigned to 6"-O-acetylgenistin by mass spectrum  $m/e$  474 ( $M^+$ ) and by elemental analysis. The analysis found: C, 57.9; H, 4.5; O, 37.5 (calculated: C, 58.2; H, 4.5; O, 37.1). The molecular formula  $C_{23}H_{22}O_{10}$  was assigned to 6"-O-acetyldaidzin by mass spectrum,  $m/e$  459 ( $MH^+$ ). Both mass spectra showed high concentrations of the ions corresponding to the aglucones, genistein ( $m/e$  270 ( $M^+$ ), figure 4A) and daidzein ( $m/e$  255 ( $MH^+$ ), figure 4B). The results obtained by pmr spectroscopy and ms also indicated that the additional acetyl group present in the two new compounds was attached to the glucose molecule rather than to the parent isoflavonoid nucleus. The data obtained by pmr, ir, uv, and ms agree with those reported by Ohta et al. (1979, 1980) for these compounds and confirm the presence of 6"-O-acetylgenistin and 6"-O-acetyldaidzin in toasted defatted soyflakes.

## Comparison of Extraction Methods

The chromatograms obtained by HPLC analysis of the soyflake extracts with (a) acetone-HCl and (b) 80% methanol are shown in figures 2 and 5, respectively. A marked difference is observed between the two chromatograms. In figure 5, peaks corresponding to 6"-O-acetyldaidzin and 6"-O-acetylgenistin (peak numbers 3 and 4 in figure 2) are almost nonexistent. Toasted defatted soyflakes contained 52 ppm of 6"-O-acetyldaidzin and 88 ppm of 6"-O-acetylgenistin. The concentrations of 6"-O-acetylgenistin and 6"-O-acetyldaidzin in the 80% methanol extract (figure 5) were 15% and 9%, respectively, of the concentrations present in the acetone-HCl extract (figure 2). The concentrations of genistin and daidzin extracted with both solvents were essentially the same. Obviously, there is selective extraction of the isoflavones with 80% methanol.

Eldridge (1982a, b) did not report the presence of the acetylisoflavones in 80% methanol extracts of a number of soy products. The difference between the two extraction solvents observed in this experiment can explain why 6"-O-acetyldaidzin and 6"-O-acetylgenistin were not detected by Eldridge (1982a, b). Glycitein and glycitin were reported to be present in a variety of soybean samples extracted with 80% methanol. The compounds were not detected in our extracts obtained with (a) acetone-HCl or (b) 80% methanol. The isoflavone content of soybeans is known to be affected by environmental conditions (Eldridge and Kwolek, 1983); thus, it is possible that glycitein and/or glycitin

were not present in our samples.

Naim et al. (1973) were the first to report glycitin and glycitein in soybeans. Extraction of glycitin and glycitein was performed by using ether followed by absolute methanol in a Soxhlet apparatus (Naim et al., 1974). Although a direct comparison of our method of extraction with that used by Naim et al. (1974) cannot be made, the important difference between the two methods was a cold extraction solvent used in this study in contrast to a hot extraction solvent used by Naim et al. (1974). The hot extraction conditions could modify the isoflavonoid constituents. Therefore, a comprehensive comparison of extraction solvents used by the various researchers should be carried out.

The presence of 6"-0-acetylgenistin and 6"-0-acetyl daidzin in toasted defatted soyflakes has been confirmed. However, environmental factors (Alexander and Watson, 1951; Eldridge and Kwolek, 1983) varietal differences (Eldridge and Kwolek, 1983; Murphy, 1932), and infections of the plants by bacteria, fungi, or insects (Loper and Hanson, 1964; Francis and Millington, 1971) affect the isoflavonoid content of soybeans. The extraction solvent was also shown in this experiment to be an important factor, especially when analyzing samples containing low concentrations of the acetyl isoflavones. Modification of isoflavones also has been reported during the fermentation of soybeans (Gyorgy et al., 1964), pasture plants (Pieterse and Andrews, 1956) and the drying of clover (Alexander and Watson, 1951). Because raw soybeans were not analyzed in this experiment or by Ohta et al. (1979, 1980) for the acetyl isoflavones, the natural occurrence of these compounds in

soybeans still remains to be investigated. Glycitein and/or glycitin have not been isolated nor chemically identified since the original report (Naim et al., 1973). Thus, the natural occurrence of glycitin and glycitein also requires further substantiation.

The estrogenic activity of glycitein, glycitin, and the two acetyl isoflavones has not been investigated. Further experimentation is required to determine the importance of these compounds in soybeans and/or soybean products intended for human use.



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Table 1. R<sub>f</sub>-values for soybean isoflavones on silica gel G

Solvent <sup>a</sup>	Acetyl daidzin	Acetyl genistin	Daidzin	Genistin	Daidzein	Genistein
A	0.19	0.30	0.05	0.13	0.69	0.76
B	0.13	0.20	0.06	0.08	0.59	0.73
C	0	0	0	0	0.12	0.41
D	0.74	0.78	0.46	0.50	0.90	0.92

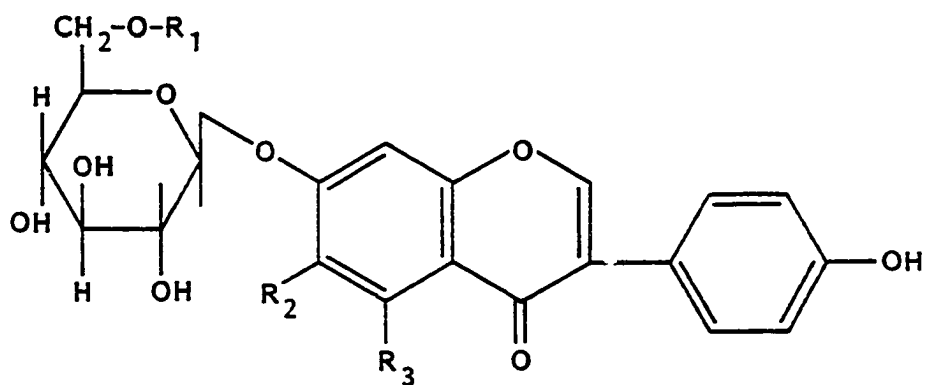
<sup>a</sup>Solvent compositions appear in materials and methods.

Table 2. UV absorption maxima (nm) of acetylgenistin and acetylaidzin

Reagent	6"-O-acetylgenistin	6"-O-acetylaidzin
Methanol	260	256
Methanol/Na-methoxide	276	258
Methanol/AlCl <sub>3</sub> :HCl	272	256
Methanol/Na-acetate	260	256

Table 3.  $R_f$ -values for sugar standards and unknown sugars

Sugar	$R_f$ -value
Glucose	0.40
Fructose	0.47
Xylose	0.52
Ribose	0.64
Rhamnose	0.68
Sugar (obtained by acid hydrolysis)	0.39
Sugar (obtained by enzyme hydrolysis)	0.00



$R_1-H;$	$R_2-H;$	$R_3-H;$	<b>Daidzin</b>
$R_1=H;$	$R_2-OCH_3;$	$R_3-H;$	<b>Glycitin</b>
$R_1-H;$	$R_2-H$	$R_3-OH;$	<b>Genistin</b>
$R_1-COCH_3$	$R_2-H$	$R_3-H;$	<b>6''-O-Acetyldaidzin</b>
$R_1-COCH_3$	$R_2-H$	$R_3-OH$	<b>6''-O-Acetylgenistin</b>

Figure 1. The structures of isoflavonoid glucosides

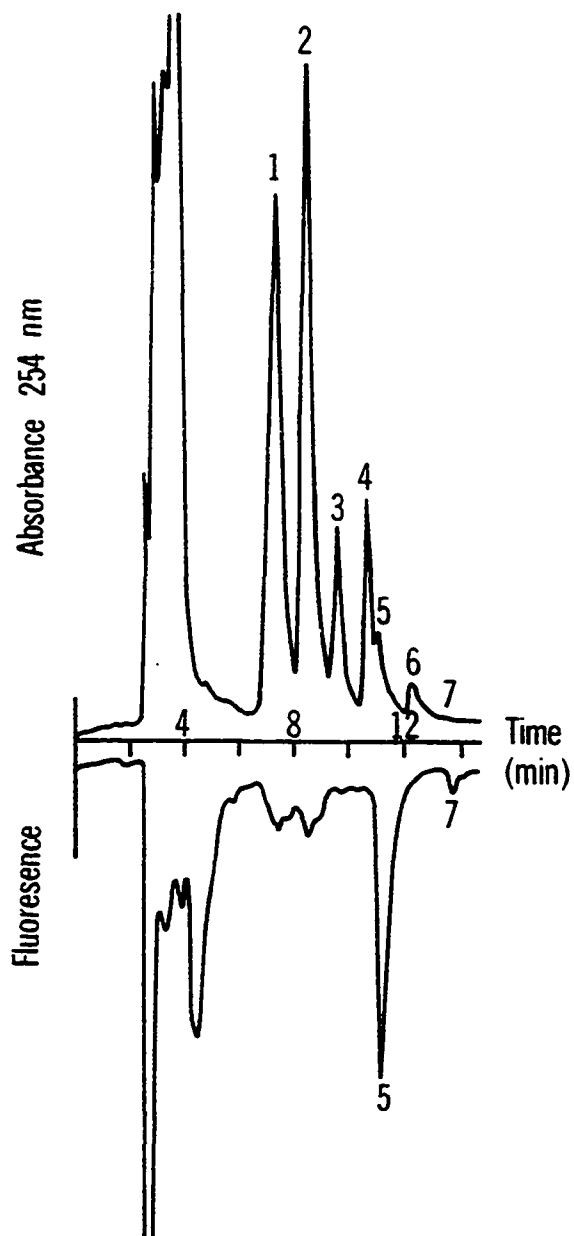


Figure 2. HPLC chromatogram of toasted defatted soyflakes extracted with acetone-HCl. Peaks: 1 = daidzin; 2 = genistin; 3 = 6''-O-acetyldaidzin; 4 = 6''-O-acetylgenistin; 5 = daidzein; 6 = genistein; 7 = coumestrol

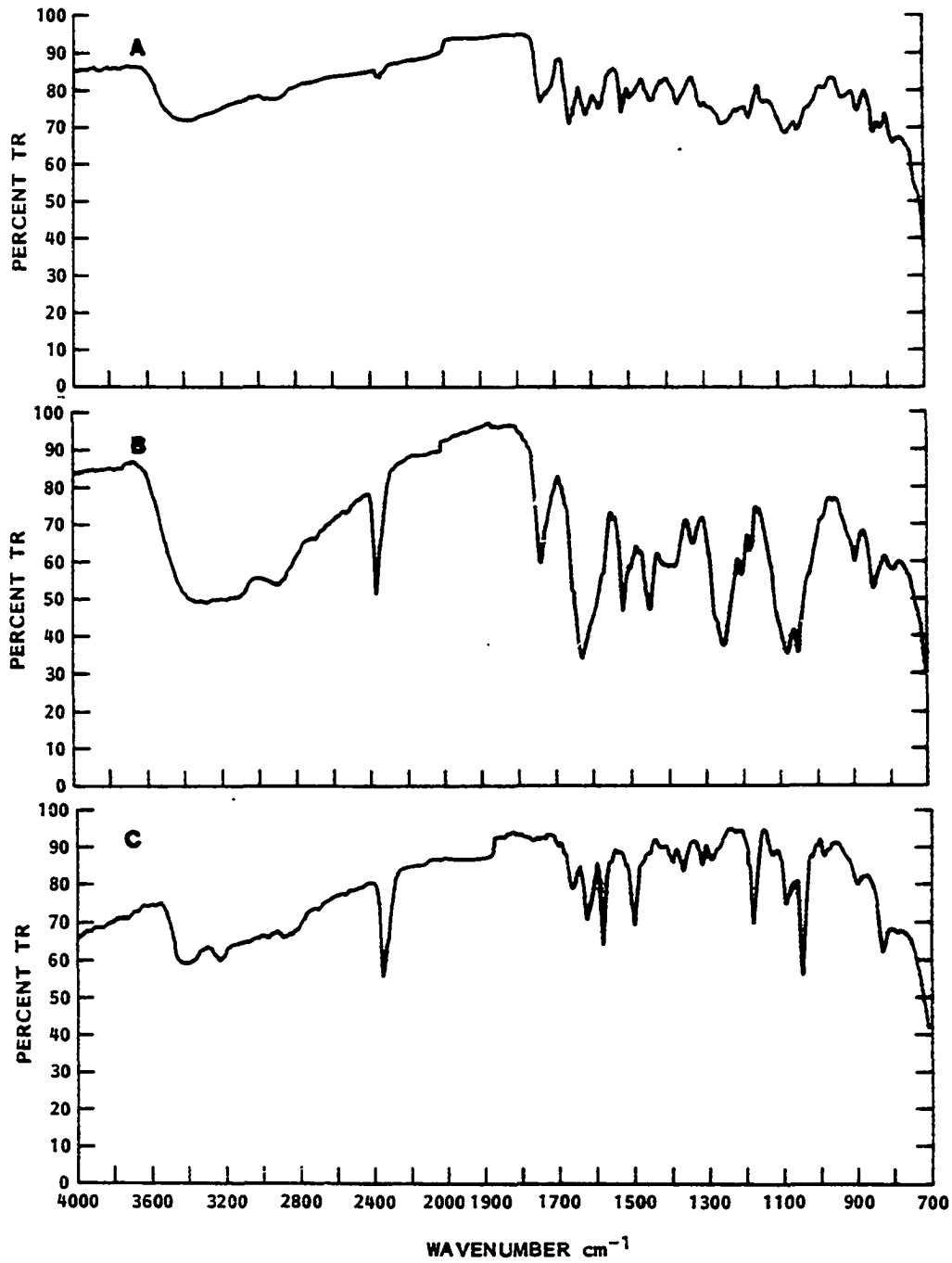


Figure 3. Infrared spectrum of A. 6''-O-acetylgenistin; B. 6''-O-acetyldaidzin; C. Genistin



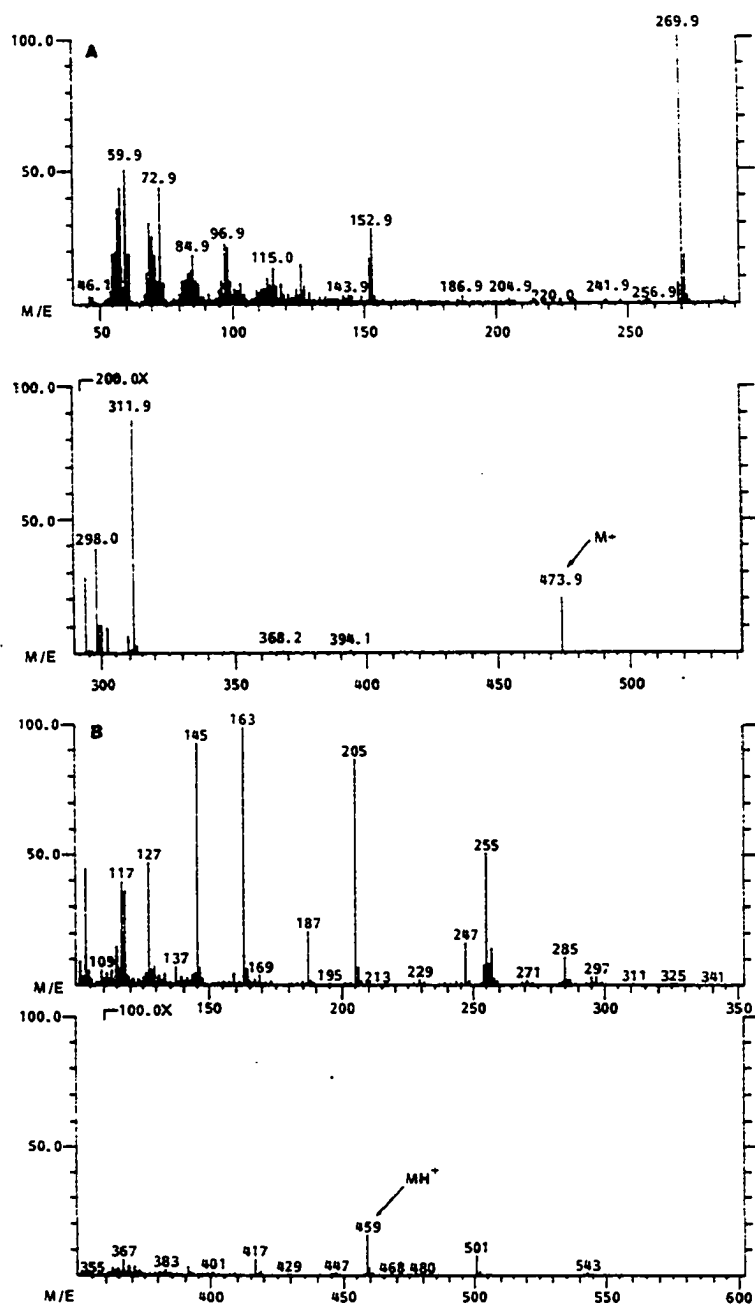


Figure 4. Mass spectrum of A. 6''-O-acetylgenistin; B. 6''-O-acetyldaizin

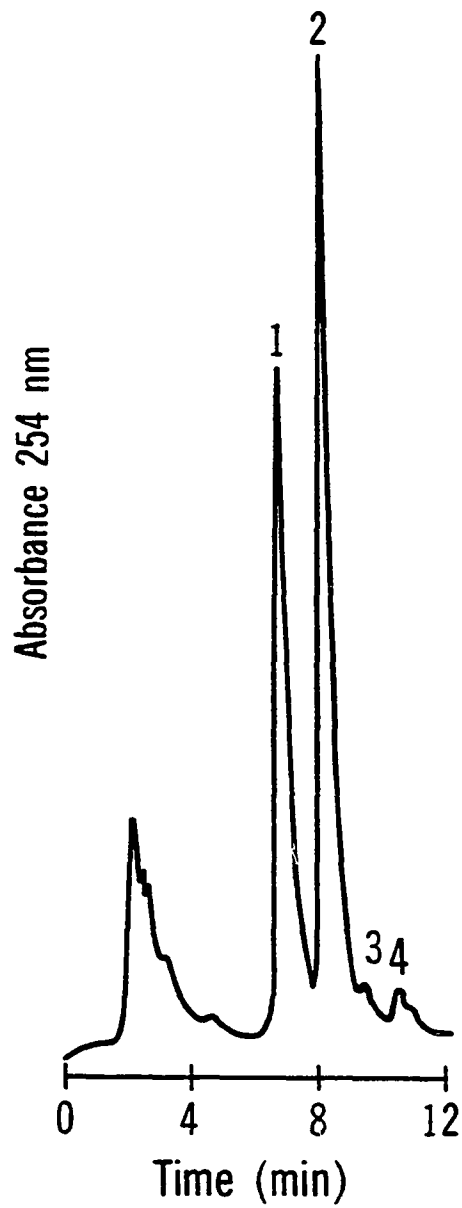


Figure 5. HPLC chromatogram of toasted defatted soyflakes extracted with 80% methanol. Peaks: 1 = daidzin; 2 = genistin; 3 = 6"-O-acetyldaidzin; 4 = 6"-O-acetylgenistin

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## SUMMARY

Phytoestrogens are widely distributed in the plant kingdom. Inevitably they find their way in human foods. Although the isoflavones genistein and daidzein are  $10^5$  times less effective estrogens than diethylstilbestrol, the compounds were found to cause infertility in sheep. Most of the information available on the isoflavones is mainly concerned with the adverse effects they have on the reproductive system of sheep. The main isoflavonoid constituents of soybeans are genistin and daidzin, glucoside forms of genistein and daidzein respectively. The estrogenic potency of genistin and daidzin has not been investigated. The use of soybean protein in human foods is increasing. There is a significant carryover of these compounds into soybean products intended for human use. Therefore, information on the possible adverse effects the isoflavones might present to human health is required.

The objectives of this study were:

1. Develop a method utilizing preparative high-performance liquid chromatography (HPLC) for the isolation and purification of the soybean isoflavones genistin and daidzin.
2. Use the purified isoflavones obtained above in feeding experiments with mice to determine their estrogenic potency.
3. Isolate and characterize two other isoflavones observed to be present in a number of soyproducts examined earlier in our laboratory.

A semi-preparative HPLC method was developed on a reverse-phase  $C_{18}$

column using a non-linear methanol-water gradient. Purification of the crude soybean extract using a silica gel column was required prior to fractionation and purification of the isoflavones using HPLC. The main limitation of the method is the solubility of the compounds in methanol which is only 5 mg/ml. A maximum concentration of 10 mg of a mixture of genistin and daidzin could be fractionated at any one time and recovery of pure compounds was 30%. Conventional chromatographic methods currently in use offer greater sample capacity. However, use of preparative HPLC significantly decreases the time required for isolation and purification of the compounds.

A significant difference in estrogenic response to the isoflavones genistein, genistin, daidzin and the synthetic estrogen diethylstilboestrol (DES) was observed between outbred and inbred strains of mice. The inbred strains B6D2F1 and B6C3F1 were more sensitive to orally administered estrogen than the outbred ICR and CD-1 strains. A slight difference between the CD-1 and ICR strains of mice was also observed. ICR was more sensitive to estrogen than the CD-1 strain. The results obtained with the different strains of mice showed that a standardization of the estrogen bioassay with respect to the mouse strain is required before comparisons between researchers using different strains of mice can be made.

The inbred strain B6D2F1 mouse was used to determine the estrogenic potency of genistin and daidzin. The estrogenic response to 1.5 mg of genistin was found to be equivalent to 1 mg of genistein. The estrogenic response to 3.8 mg of daidzin was equivalent to 1 mg of

genistein. The relative potencies of genistin and daidzin were 0.66 and 0.26 respectively. A 1:1 molar equivalency in estrogenic potency between genistein and genistin was observed. Extrapolation of the data showed that a dose of 8,000 ppm in the human diet/day present as genistin would produce an estrogenic effect in humans. The metabolism of isoflavones differs in animal species thus, humans can be more sensitive or alternatively less sensitive than mice to the phytoestrogens. Concentrations of phytoestrogens lower than those required for an estrogenic effect have also been reported to interfere with reproduction in mice. Phytoestrogens at low levels can act as antiestrogens, and in this respect they could have a beneficial effect as related to hormone dependent mammary tumors. Genistin and daidzin exhibit an estrogenic response in mice. The metabolism of these compounds and their possible biological effects in humans have not been investigated. Their possible adverse or beneficial effects in relation to human health should be investigated.

The compounds 6"-O-acetylgenistin and 6"-O-acetyldaidzin have been isolated from toasted defatted soyflakes and their structure was confirmed using ultraviolet, infrared, nuclear magnetic resonance, mass spectroscopy, acidic and enzymatic hydrolysis. The use of 80% methanol was found to be ineffective in extracting the acetyl isoflavones. The extraction solvents used by the various researchers in the field are different and reports on the native isoflavonoid constituents in soybeans are confusing. A standardization of the method of extraction is required. Environmental factors, varietal differences, infections of

the plants by fungi, bacteria or insects affect the isoflavonoid content of soybeans. Modification of isoflavones also occurs during the fermentation of soybeans. Raw soybeans have not been analyzed for the presence of these compounds. Thus the natural occurrence of 6"-0-acetyldaidzin and 6"-0-acetygenistin in soybeans still remains to be investigated. Quantitative information on the acetyl isoflavones, is not available. A comprehensive survey of soy protein products is required to determine if the compounds are affected by processing. The estrogenic activity of these compounds has not been investigated. In view of the greater use of soy-protein products in the world basic information is necessary to determine the importance of these compounds in the human diet.

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