ANALYSIS OF *IN VITRO* BINDING OF DIETARY FIBERS BY THE PHYTOESTROGEN, DAIDZEIN, IN THE PRESENCE AND ABSENCE OF IRON

by

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ABSTRACT

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Analysis of *In Vitro* Binding of Dietary Fibers by the Phytoestrogen, Daidzein, in the Presence and Absence of Iron

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The concentration of unbound daidzein, a phytoestrogen, found in soy products, was measured by high performance liquid chromatography in solutions containing insoluble (cellulose) and soluble (locust bean gum) dietary fibers *in vitro*. Iron was added to selected samples containing daidzein in solution with insoluble and soluble dietary fibers. Fiber samples were incubated at 37° C during hydration and exposed to pH adjustments to mimic pH changes present in the human digestive system.

There were significant differences between the concentration of unbound daidzein in samples containing soluble fiber compared to insoluble fiber ($p \leq 0.001$). The addition

of iron did not affect the binding of daidzein to either fibers, *soluble* fibers or *insoluble* fibers ($p \leq 0.630$ and $p \leq 0.092$), when compared to the fibers alone. The results suggest that fibers may potentially modify the bioavailability of the isoflavonic compound for absorption.

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Most of all, I am extremely grateful to my family, my parents, brothers and sister. Mom, thank you for everything. To my husband, Bill, and children, Ashley, Amanda, and Ethan, I love you with all my heart!

I dedicate this thesis to my brother, Michael, and my sister, Kennice, who are no longer with us. We miss you, dearly!

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INTRODUCTION

Extensive research has shown that there is a strong association between diet and the incidence of disease-related conditions. Certain naturally-occurring nonnutrient compounds have the potential to prevent or delay the onset of chronic diseases in humans and animals (Guhr and LaChance 1997). These natural compounds, called *phytochemicals,* are present in fruits, vegetables, grains, legumes, herbs, and seeds (Guhr and LaChance 1997).

Among the phytochemicals is a broad class of nonsteroidal estrogens called *phytoestrogens*. Phytoestrogens, also known as plant estrogens or dietary estrogens, are classified into three major categories: isoflavones, resorcyclic acid lactones, and coumestranes (Bannwart et al. 1984). Isoflavones are the major phenolic compounds found in soybeans (Zhou and Erdman 1997).

The isoflavones, genistin and daidzin, as well as, their aglucones (non-glucoside forms), genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7, 4'-dihydroxyisoflavone) are the primary isoflavones present in soybeans. ß-glycoside forms of isoflavones are transformed into their active aglucone forms by bacterial enzymes in the intestine (see review by Setchell 1998). Active forms of isoflavones reach concentrations in *biological fluids* surpassing that of the principal endogenous mammalian estrogens, estradiol or estrone (see review by Setchell 1998). Estradiol is the principle estrogen produced in human female ovaries (Montgomery et al. 1996).

The enzymatic cleavage of genistin and daidzin by ß-glucosidase produces the aglucones, genistein and daidzein, respectively (Zhou and Erdman 1997). The conversion of daidzein to equol is the result of further bacterial degradation (Setchell et al. 1984).

Phytoestrogens, structurally similar to human estrogen, can act as estrogen mimics by exhibiting weak binding to estrogen receptors (ER) (Zhang, Song et al. 1999, Tew et al. 1996, and Martin et al. 1978). The phenolic ring of the phytoestrogen molecule mediates binding to estrogen receptors (see review by Setchell 1998). This involvement with estrogen receptors may initiate physiological changes to cells, tissues, or organs. Phytoestrogens like human estrogen have the potential to function as either estrogenic or antiestrogenic compounds depending on the amount that is ingested and absorbed by the body (Martin et al. 1978). A phytoestrogen's estrogenic or antiestrogenic activity is due to but not limited to "concentration dependency, receptor status, presence or absence of endogenous estrogens, and type of target organ or cell" (see review by Setchell 1998).

The ability of a phytoestrogen to act as an estrogenic or antiestrogenic compound is an important factor in determining whether it would have a positive or negative impact on health. While phytoestrogens can display weak estrogen action, they can also act as an antiestrogen as shown by competitive binding with human estrogen at highly specific receptor sites (see review by Setchell 1998). Human estrogen is important for normal sexual development and maintenance of a healthy reproductive system in females.

The antiestrogenic activity of phytoestrogens may contribute to a reduction in hormone-dependent cancers (Adlercreutz et al. 1993, Peterson and Barnes 1991, and Troll et al. 1980). According to Adlercreutz et al. (1991), selective phytoestrogens have

an *antiproliferative activity* on breast cancer cells, as well as, an inhibitory effect on protein tyrosine kinase activity.

Isoflavones are reported to have a number of disease-fighting properties; such as, an antitumor effect in breast cancer (Barnes 1995), a protective role in hormone-dependent cancers (Barnes 1995, Adlercreutz et al. 1995, and Setchell et al. 1984), an inhibition of cancer cell growth and angiogenesis, which is the development of blood vessels around a tumor (Fotsis et al. 1995), and an effect on sex hormone metabolism (Adlercreutz et al. 1995).

Ingestion of isoflavones has been associated with a decrease in the incidence of coronary heart disease (Anderson et al. 1995). Isoflavones with antioxidant properties have the potential to inhibit cardiovascular disease (see review by Lichtenstein 1998 and Wang et al. 1995). Isoflavones have been shown to exhibit weak antioxidant properties, thereby inhibiting the formation of carcinogens in the intestine (Adlercreutz et al. 1991). Studies in animals have suggested that isoflavones have been instrumental in decreasing plasma low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol in males and females, as well as increasing plasma high density lipoprotein cholesterol in females without any deleterious effects on the reproductive system (Anthony et al. 1996; see review by Carroll and Kurowska 1995). Wang et al. (1995) reported a reduction in plasma LDL cholesterol concentrations and an increase in plasma HDL cholesterol concentrations in human hypercholesterolemic females. However, not all reports have verified these positive outcomes (Gooderham et al. 1996 and Grundy and Abrams 1983).

Phytoestrogens affect the reproductive tract and reproductive cycles of animals (Bennetts, Underwood, and Shier 1946). A syndrome first identified in agricultural

animals in Australia, known as the *Clover disease,* causes these animals to become infertile as a result of phytoestrogen intake from clover and other grasses (Bennetts, Underwood, and Shier 1946). Animal studies have also found that phytoestrogens may cause sexual developmental disorders. High levels of phytoestrogens in young female rats result in *premature anovulatory syndrome* characterized by suppression of ovulation (Whitten et al. 1995). Similarly high levels of phytoestrogens are associated with altered male sexual development in rat pups (Whitten et al. 1995). Some studies show that there may be potential adverse effects in infants fed soy-based formulas (Irvine, Fitzpatrick, and Alexander 1998 and Irvine et al. 1995).

A projection based on animal studies (Wang and Murphy 1994), suggests that humans need to consume 1.5 to 2.0 mg of soy isoflavones per kg body weight per day to gain a protective health benefit.

Soybeans contain up to 1 to 3 mg of isoflavones per gram, principally as genistin and daidzin (Adlercreutz et al. 1995). Processed soy foods like tempeh, tofu, miso, soy protein isolate, soy milk, and soy flour are also good sources of isoflavones (Wang and Murphy 1994). Not all soy foods have the same isoflavone content (Wang and Murphy 1994).

Since plant-based diets may be high in phytoestrogens and dietary fiber, it is important to determine whether dietary fiber interferes with or enhances the bioavailability of phytoestrogens for absorption. Studies have shown that dietary fiber binds to phytoestrogens (Tew et al. 1996) and human estrogens (Rose et al. 1991), thus decreasing the bioavailability of these compounds. Lampe et al. (1998) reported that dietary fiber may enhance the production of bacterial enzymes necessary to convert the isoflavone, daidzein, to equol.

The interaction between dietary fiber and phytoestrogens has not been thoroughly investigated. This study investigates differences in the concentration of unbound phytoestrogen, daidzein, between samples containing soluble dietary fiber (locust bean gum) and an insoluble dietary fiber (cellulose) in the presence and absence of iron.

Statement of Problem

The purpose of the study is to determine the effect of soluble (locust bean gum) and insoluble (cellulose) dietary fibers with and without the presence of iron on the concentration of unbound daidzein as measured by high performance liquid chromatography.

Null Hypothesis

There is no statistically significant difference in the concentration of unbound daidzein in solution between samples containing soluble (locust bean gum) and insoluble (cellulose) dietary fibers with and without the presence of iron.

REVIEW OF LITERATURE

Introduction

Isoflavones are nonnutrient compounds found in plants. Many plant-derived foods, especially soy products, are excellent sources of isoflavones (Wang and Murphy 1994). Asian people have consumed soy products for thousands of years, but only in the last two decades has an increase in soybean consumption been observed in the West. Messina and Messina (1991) have suggested that the recent increase in soy product consumption in the West is attributed to several factors; such as, "economics, health, ethics, and the environment."

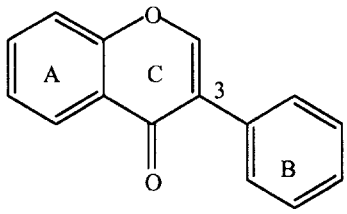
Though isoflavones neither provide energy nor building material for the body they have the potential to perform other important beneficial functions. A selective literature review of isoflavones will highlight their chemical structures, their absorption and metabolism, their reported anti-tumor properties, their effects on the reduction of the risk of cardiovascular disease and their potential adverse effects. The review will continue with an examination of the properties significance, structure, and interrelationship of dietary fiber and iron, as well as, high performance liquid chromatography.

Chemical Structures

Isoflavones are phenolic compounds found in plants. The structure of isoflavones differ from the structure of other flavonoids in the position of the benzene ring

(Wang and Murphy 1994). The benzene B-ring of an isoflavone is linked to carbon 3 of the C-ring instead of carbon 2 (See Figure 1) as observed in flavonols.

Isoflavone



Flavonol

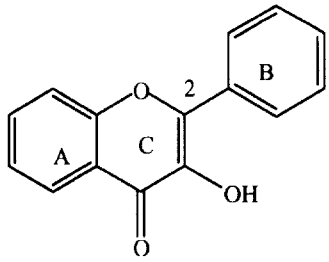
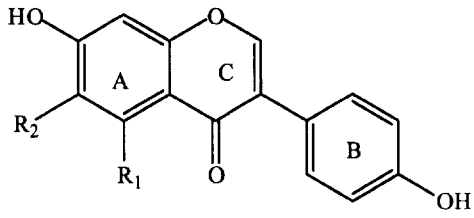


Figure 1. A comparison of the chemical structure of an isoflavone and a flavonol (Smith and Yang 1994).

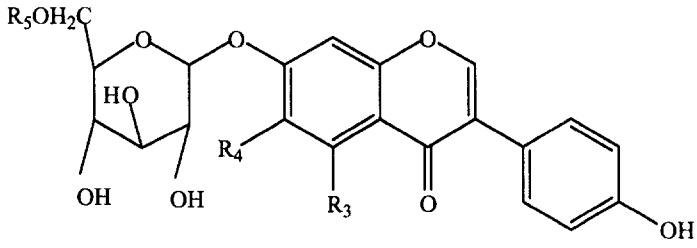
Dietary isoflavones can exist either in combination with ß-D-glucose as a glycoside or as a free non-glycoside form. The principal isoflavone glycosides extracted from soybeans are genistin and daidzin. A third soy isoflavone glycoside called glycitin, is also present but in a much lower concentration than genistin and daidzin (Zhang, Wang et al. 1999). Glycoside forms of isoflavones that have been identified in extracts of soybeans, include: daidzin, genistin, glycitin, 6"-O-Acetyldaidzin, 6"-O-Acetylgenistin, 6"-O-Acetylglycitin, 6"-O-Malonyldaidzin, 6"-O-Malonylgenistin, 6"-O-Malonylglycitin (Wang and Murphy 1994). The corresponding non-glycoside forms of the soy isoflavones are genistein, daidzein, and glycitein. The non-glycoside forms of isoflavones contain a three ring structure, whereas the glycoside forms possess a four ring structure. Structures of the known isoflavones are shown on the following page (See Figure 2).

Non-glycosides



R_1	R_2	Compounds
H	H	daidzein
OH	H	genistein
H	OCH_3	glycitein

Glycosides



R_3	R_4	R_5	Compounds
H	H	H	daidzin
OH	H	H	genistin
H	OCH_3	H	glycitin
H	H	$COCH_3$	6"-O-Acetyldaidzin
OH	H	$COCH_3$	6"-O-Acetylgenistin
H	OCH_3	$COCH_3$	6"-O-Acetylglycitin
H	H	$COCH_2COOH$	6"-O-Malonyldaidzin
OH	H	$COCH_2COOH$	6"-O-Malonylgenistin
H	OCH_3	$COCH_2COOH$	6"-O-Malonylglycitin

Figure 2. Chemical structures of 12 isoflavone isomers (Wang and Murphy 1994).

Isoflavones are very similar in structure to endogenous estrogens found in humans and other animals. According to Setchell and Adlercreutz (1988), "the distance between the two aromatic hydroxyl groups on the nucleus of the isoflavones is almost identical to the distance between the C-3 and C-17 hydroxy groups of oestradiol" (See Figure 3). It is the C-3 phenolic group that is required for estrogenic activity (see review by Setchell and Adlercreutz 1988).

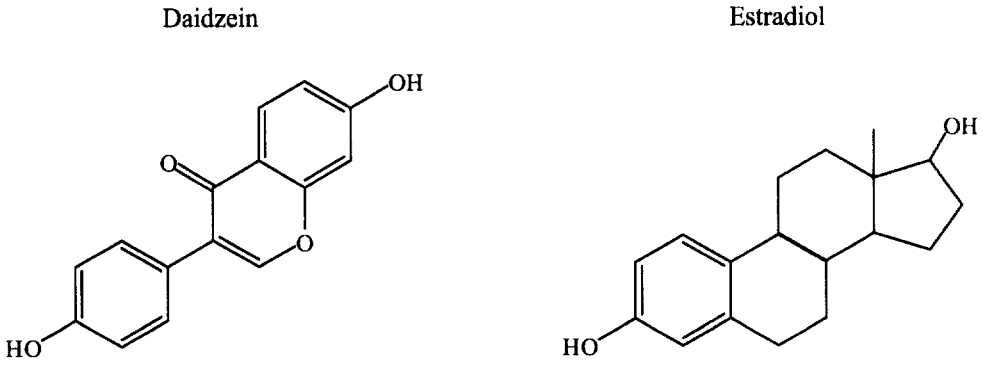


Figure 3. Chemical structure of daidzein (an isoflavone) and estradiol.

Metabolism and Absorption of Isoflavones

The isoflavone glycosides, genistin and daidzin, are hydrolyzed to the non-glycoside (free) forms in the lower small intestine by bacterial ß-glucosidase enzymes. The enzymatic cleavage of genistin and daidzin by ß-glucosidase yields the non-glycoside forms, genistein and daidzein, respectively (Zhou and Erdman 1997) (See Figure 4). Though numerous species of bacteria produce ß-glucosidases, it is the *Lactobacilli*, *Bacteroides*, and *Bifidobacteria* that are largely responsible for cleaving the glycosidic bond within the small intestine of mammals (Hawksworth, Drasar, and Hill 1971).

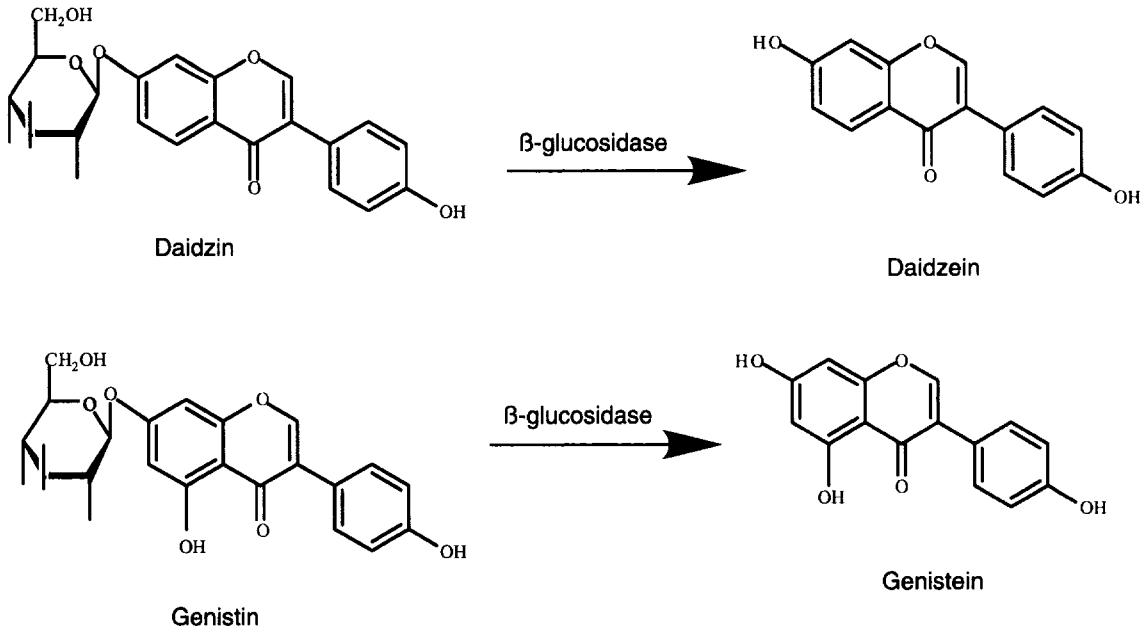


Figure 4. Conversion of daidzin and genistin to daidzein and genistein by ß-glucosidase.

Daidzein may either be absorbed (Setchell and Cassidy 1999) or further metabolized to various isoflavone metabolites in the intestine, including equol (Axelson et al. 1982) and O-Desmethylangolensin (Setchell and Adlercreutz 1988). See Figure 5 for two proposed metabolic pathways for daidzein.

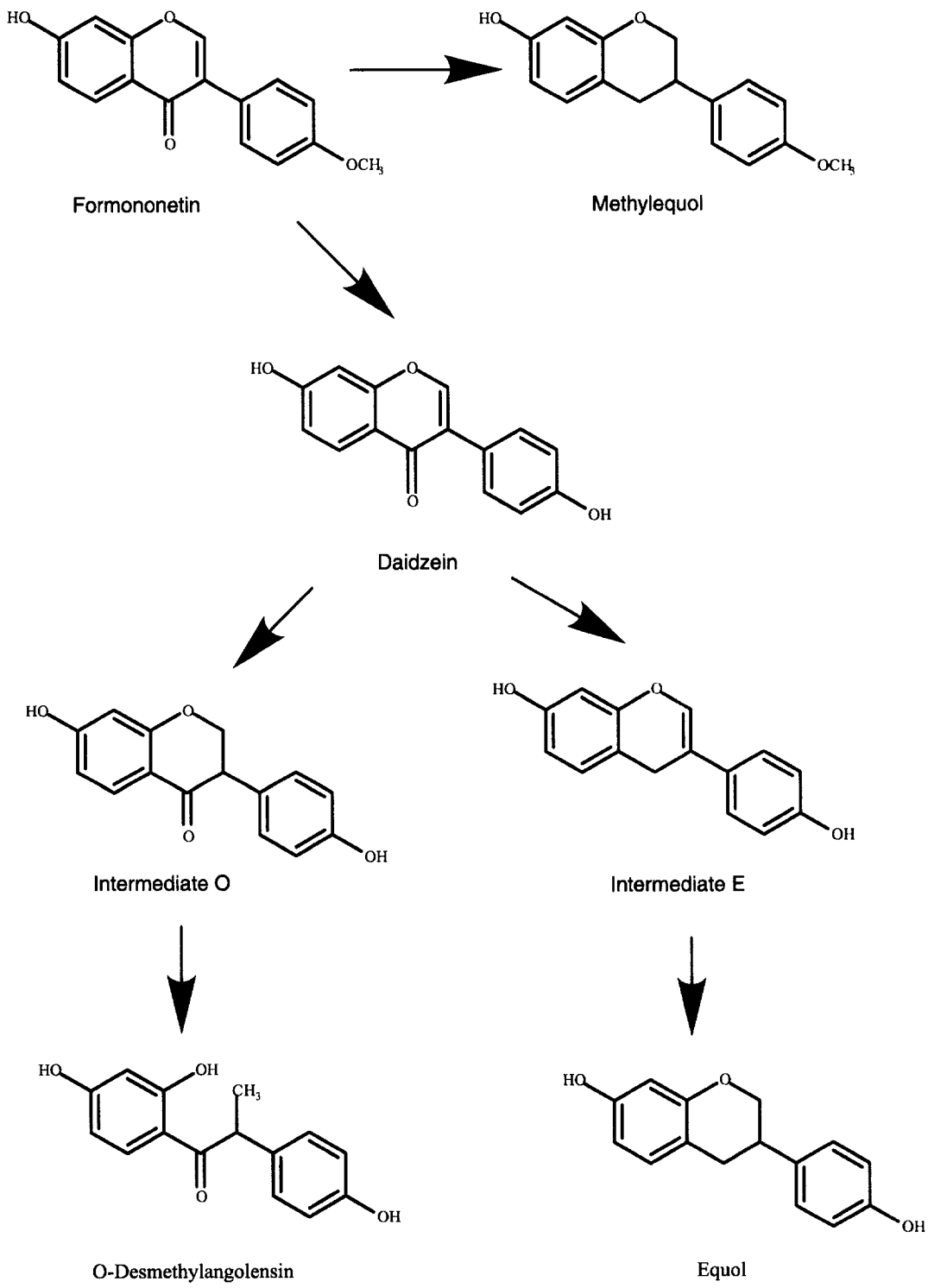
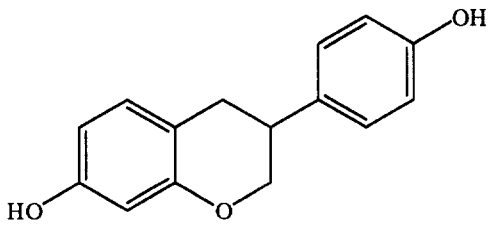


Figure 5. Chemical structures and proposed metabolic pathways of dietary isoflavones in man and animals (Setchell and Aldercreutz 1988).

Equol is a phytoestrogen that possesses weak estrogenic activity when compared to the human estrogen, estradiol (Setchell 1984). Shutt and Cox (1972) reported that equol has from 10^{-3} to 10^{-5} times the estrogenic activity of estradiol-17ß. Tang and Adams (1980) found in a study using animals that equol and estradiol-17ß compete for the same cytoplasmic estrogen receptors. Equol is similar in structure to estradiol (See Figure 6) and is a more potent estrogen mimic than daidzein (Setchell et al. 1984). Equol is thought to be one of the isoflavone compounds considered responsible for the infertility syndrome of sheep known as *Clover disease* (Shutt and Braden 1968).

Equol



Estradiol

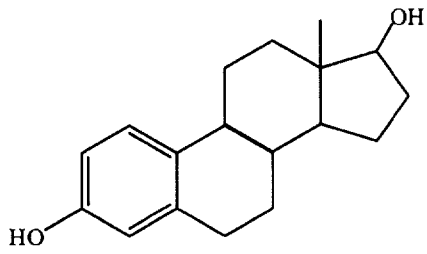


Figure 6. A comparison of the chemical structures of equol and estradiol (Setchell et al. 1984).

Axelson et al. (1982) first identified equol in human urine. The results of two studies with humans (Xu et al. 1995, Xu et al. 1994) and one with animals (King 1998) suggest that intestinal bacteria metabolize and degrade soybean isoflavones. Equol, a metabolite of daidzein, is probably formed by gut microflora in humans (Axelson et al. 1982). Not all humans are able to convert daidzein to equol, possibly related to individual differences in the complement of intestinal bacteria (Lampe et al. 1998).

The isoflavone non-glycosides, daidzein and genistein, are more readily absorbed in the intestine than the glycoside forms (Xu et al. 1995). Song et al. (1998) stated that

the glycoside form of isoflavones is not absorbed in the intestine unless hydrolysis of the glycosidic bond occurs. According to Xu et al. (1995), the glycoside forms are less bioavailable than the non-glycoside forms due in part to their *higher hydrophilicity and greater molecular weight.* The bacteria in the intestinal tract determine the *magnitude and pattern of isoflavone bioavailability* (Xu et al. 1995).

Recent studies have shown that daidzein is more bioavailable than genistein in animals (King 1998) and humans (Xu et al. 1995 and Xu et al. 1994). In contrast, King and Bursill (1998) found that the bioavailabilities of daidzein and genistein in human subjects were similar, despite a higher urinary excretion of daidzein compared to genistein. Zhang, Wang et al. (1999) reported that the bioavailability of daidzein and genistein are similar in men and women.

Research indicates that once non-glucoside isoflavones are absorbed by the intestinal *mucosa*, they soon undergo glucuronidation (Zhang, Song et al. 1999 and Sfakianos et al. 1997). Isoflavones contain one or more hydroxyl groups that can react with carbon 1 of glucuronic acid to form a glucuronide. Daidzein has hydroxyl groups in positions 7 and 4. An *in vitro* study found that the hydroxyl group in position 7 was most likely to react with uridine 5'-diphosphoglucuronic acid to produce isoflavone glucuronides (Zhang, Song et al. 1999).

Research indicates that isoflavone glucuronides may be synthesized in two locations. Sfakianos et al. (1997) reported that when genistein was infused into rat duodenum, the compound *was rapidly absorbed from the intestine, taken up by the liver and excreted into the bile as its 7-O-ß glucuronide conjugate.* Similarly, after absorption by the intestinal tract, equol was conjugated to glucuronic acid in the liver of animals (Shutt, Weston, and Hogan 1970). When genistein was infused into the hepatic portal

vein of rats, it also was taken up by the liver, *conjugated with glucuronic acid and transported into the bile* (Sfakianos et al. 1997). Free isoflavones are transported to the liver via the portal vein, where they are acted upon by UDP-glucuronyltransferase and sulfotransferase to form glucuronide and sulfate conjugates that are then excreted in the bile (Zhang, Song et al. 1999). Sfakianos et al. (1997) have suggested that *in vivo* glucuronidation may also occur in the intestinal mucosa because following duodenal infusion of genistein, the portal blood contained mainly genistein 7-O-ß glucuronide. Zhang, Song et al. (1999) have suggested that daidzein and genistein glucuronides may account for some of the *biological effects of isoflavones*.

Anti-tumor Effect of Isoflavones

Isoflavones have a potential role in cancer prevention; notably breast, colon, and prostate cancer (see review by Kennedy 1995). In the United States, breast cancer is the leading cause of death in women between 35-45 years of age, and the risk of breast cancer increases after the age of 50 (Martini and Bartholomew 1997). According to Martini and Bartholomew (1997), approximately 12% of women in the United States will develop breast cancer.

Human estrogen, the hormone responsible for normal sexual maturation and maintenance of a healthy reproductive system in females, is synthesized in the ovaries (Montgomery et al. 1996). The effects of estrogen on diverse cellular activities is mediated by binding to intracellular estrogen receptors.

Isoflavones can act either as estrogen mimics or antagonists (see review by Setchell 1998) as a result of their binding to estrogen receptor sites (Zhang, Song et al. 1999, Tew et al. 1996, and Martin et al. 1978). The antiestrogenic activity of isoflavones

is believed to decrease the incidence and growth of certain hormone-dependent cancers. Isoflavones are believed to act as an antiestrogen when they bind to an estrogen receptor and physically prevent human estrogen from binding to that site. *In vitro* studies have shown that isoflavones compete with estradiol for estrogen receptors in human breast cancer cells (see review by Setchell 1998 and Martin et al. 1978) and have been shown to be antiproliferative with regard to estrogen-sensitive breast cancer cells (Peterson and Barnes 1991). Daidzein is a weaker growth inhibitor *in vitro* than genistein (Peterson and Barnes 1991).

Peterson and Barnes (1991) stated that *the presence of the estrogen receptor is not required for the isoflavones to inhibit tumor cell growth.* The results from their *in vitro* studies suggested that the effects of isoflavones may be independent of the estrogen receptor pathway (Peterson and Barnes 1991). However, Peterson and Barnes (1991) noted that this finding does not *rule out the involvement of ER* (estrogen receptor) *in isoflavone action in ER+* (estrogen receptor activated) *cells.*

Soybean diets have been shown to decrease breast tumor incidence in animal models (Hawrylewicz, Huang, and Blair 1991, Baggott et al. 1990, Barnes et al. 1990, and Troll et al. 1980). Barnes et al. (1990) fed casein-soybean protein chips (PSC) to weanling rats injected with N-nitrosomethylurea, a direct-acting carcinogen, and found that the incidence of mammary tumors decreased with increasing concentration of PSC. The authors attributed the inhibition of tumor growth to isoflavones present in the soybean protein (Barnes et al. 1990). Hawrylewicz and coworkers (1991) reported that casein-fed rats had approximately 200% greater total tumor weight than soybean protein isolate-fed rats. The soybean protein isolate (SPI) diet fed to rats decreased the incidence of mammary tumors from 80% to 42% (Hawrylewcitz, Huang, and Blair 1991).

However, when the SPI diet was fortified with methionine, there was an increased incidence of mammary tumors (Hawrylewcitz, Huang, and Blair 1991). Methionine is an amino acid precursor for polyamine synthesis (Montgomery et al. 1996). Hawrylewcitz et al. (1991) reported that *intracellular polyamine concentrations increase during tumor cell proliferation.*

Research indicates that isoflavones inhibit human aromatase (Adlercreutz et al. 1993 and Kellis and Vickery 1984). Human aromatase is a cytochrome P-450 enzyme that converts *androgens to estrogens in many tissues* (Adlercreutz et al. 1993). When an isoflavone inhibits aromatase it may bring about a decrease in the concentration of circulating estrogens (Ingram et al. 1997). Lower concentrations of circulating estrogens have been observed in the plasma and urine of Oriental immigrant women compared to Caucasian American women who have consumed a traditional Western diet (Goldin et al. 1986). According to Goldin et al. (1986), higher levels of estrogen in the urine and plasma are thought to be related to an increased risk of human breast cancer.

The typical Western diet is characterized by high levels of fat and protein and low levels of fiber and complex carbohydrates. Adlercreutz et al. (1991) suggested that the Western diet plays a potential role in hormone production, metabolism, or action at the cellular level compared to the semi-vegetarian or vegetarian diet in Asian countries. The low breast cancer mortality rates in Asian countries are thought to be partly attributed to soyfood consumption (see review by Adlercreutz et al. 1991 and Peterson and Barnes 1991).

There is evidence that isoflavonic compounds stimulate the production of sex-hormone-binding globulin (SHBG) in the liver (see review by Adlercreutz et al. 1991, Adlercreutz 1990 and Adlercreutz et al. 1987). Studies have shown that the Asian diet,

low in fat and high in fiber, is associated with low plasma and urinary sex hormone levels and high SHBG levels, which result in a decrease in the bioavailability of sex hormones (see review by Adlercreutz 1990 and Adlercreutz et al. 1987). In contrast, a typical Western diet, high in fat and low in fiber, is associated with low SHBG levels and high plasma and urinary sex hormone levels, thereby increasing the bioavailability of sex steroids that increase the risk of hormone-dependent cancers (see review by Adlercreutz et al. 1991 and Adlercreutz 1990). An increase in SHBG in the blood plasma, decreases the level of free estradiol and free testosterone in the plasma, thereby reducing the concentration of both the albumin-bound and free fractions of sex hormones in the blood plasma (see review by Adlercreutz et al. 1991). The reduction in both albumin-bound and the free fraction of sex hormones in blood plasma decreases their biological activity and the rate of metabolic clearance (see review by Adlercreutz 1995).

Akiyama et al. (1987) suggested that genistein has anticarcinogenic properties associated with tyrosine protein kinase activity. Protein kinases are enzymes *that phosphorylate intracellular proteins*; for example, tyrosine-specific protein kinase (TPK) phosphorylates tyrosine residues (Montgomery et al. 1996). Protein kinases usually act in conjunction with protein phosphatases to *regulate the activity of proteins in response to hormonal stimuli* (Montgomery et al. 1996). The tyrosine kinase activity of *src* (Rous sarcoma virus), a retroviral gene family, is stimulated by several receptor hormones and growth factors (ex. epidermal growth factor [EGF]) (Montgomery et al. 1996). When estrogen binds to EGF, it stimulates tyrosine kinase activity. Typically, EGF binding is required to activate tyrosine kinase activity of the EGF receptor protein (Montgomery et al 1996). The tyrosine-specific protein kinase activity of epidermal growth factor (EGF)

receptors, pp60$^{v\text{-}src}$, and pp110$^{gag\text{-}fes}$ kinases were inhibited *in vitro* by genistein (Akiyama et al. 1987). The effects of genistein on the system caused a *dose-dependent inhibition of autophosphorylation of the EGF receptor* (Akiyama et al. 1987). Peterson and Barnes (1991) suggested that TPK activity *may be associated with tumor cell growth and tumor recurrence.*

Studies have shown that some nonisoflavone compounds also found in soybeans have anticarcinogenic activity (see review by Messina and Messina 1991 and Messina and Barnes 1991). Protease inhibitors, phytic acid, saponins, phytosterols, and phenolic compounds are found in fairly high concentrations in soybeans (see review by Messina and Messina 1991). There is evidence that several nonisoflavonic compounds suppress carcinogenesis *in vivo,* including: inositol hexaphosphate (phytic acid), the protease inhibitor known as Bowman-Birk inhibitor, and a soybean-derived sterol (ß-sitosterol) (see review by Kennedy 1995).

Isoflavones and Cardiovascular Disease Risk

Soybeans contain many substances, in particular soy protein and soy isoflavones, that may reduce the risk of developing coronary heart disease (CHD) (Anderson et al. 1995 and see review by Adlercreutz 1990). There are several potential mechanisms by which soy isoflavones and soy protein influence human physiology and thereby CHD risk, including alterations of thyroid status and bile acid balance. In addition, some studies have suggested that soy isoflavones and soy protein display antioxidant properties (Wei et al. 1993 and Naim et al. 1976). Lichtenstein (1998) suggested that a high concentration of soy protein in the diet may displace foods that are high in saturated fat, thus having an indirect blood cholesterol lowering effect .

Animal studies have suggested that a soybean-based diet has an effect on thyroid regulation (see review by Forsythe 1995). Animal data have shown that the concentration of thyroxine in the blood, a thyroid hormone, is higher when the diet contains soy protein (see review by Forsythe 1995). Forsythe (1995) who reviewed the literature on the effect of soy protein on blood thyroxine levels, suggested that an increase in blood thyroxine preceded a decrease in blood cholesterol and that thyroxine may be involved in a mechanism for lowering blood cholesterol. A decrease in blood cholesterol is associated with a reduced risk of coronary heart disease (see review by Setchell and Cassidy 1999).

Isoflavones exhibit weak antioxidant properties, which may inhibit the formation of certain carcinogens in the intestine (Adlercreutz et al. 1991). Naim et al. (1976) reported that the antioxidant activity of isoflavones is related to the number of hydroxyl groups in the structure. An increase in the number of hydroxyl groups within the structure is positively correlated to the degree of antioxidant ability of the compound (Naim et al. 1976). Wei et al. (1993) reported that genistein inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced hydrogen peroxide formation both *in vivo* and *in vitro*. The study demonstrated that genistein is capable of *scavenging hydrogen peroxide in a cell-free system* (Wei et al. 1993). The authors suggested that the anticarcinogenic effects of soybean isoflavones are due to their antioxidant properties (Wei et al. 1993). Pratt and coworkers (1981) identified phenolic compounds in soy protein hydrolyzate (SPH) as the substances responsible for the antioxidant properties of SPH. Pratt et al. (1981) reported that flavonoids, in particular isoflavones, and phenolic acids are both *primary and synergistic antioxidants and obviously contribute to the antioxidant activity of SPH.*

Carroll and Kurowska (1995) reviewed animal and human studies which demonstrated that soy protein has hypocholesterolemic effects. A primate study has shown that isoflavones present in soy protein have been instrumental in decreasing the concentrations of plasma low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol in males and females, as well as increasing plasma high density lipoprotein (HDL) cholesterol in females without any deleterious effects on the reproductive system (Anthony et al. 1996). In a primate study, animals fed a soy protein diet rich in isoflavones had favorable blood lipid profiles, whereas animals fed a soy protein diet from which isoflavones had been removed had blood lipid profiles characteristic of animals fed a non-soy protein diet (Anthony et al. 1996). Wang et al. (1995) reported that a soybean protein diet caused a reduction in plasma LDL cholesterol concentrations and an increase in plasma HDL cholesterol concentrations in human hypercholesterolemic females. Anderson et al. (1995) found that soybean protein rather than animal protein significantly decreased plasma total cholesterol, LDL cholesterol, and triglycerides without significantly affecting plasma HDL cholesterol in hypercholesterolemic humans. In contrast, isoflavones did not alter blood lipid concentrations in normocholesterolemic humans (Hodgson et al. 1998, Gooderham et al. 1996, and Grundy and Abrams 1983).

Dietary soy protein has been reported to promote bile acid excretion in animals (see review by Potter 1996). An increase in bile acid excretion due to dietary soy protein may indirectly lower blood cholesterol concentrations (see review by Lichtenstein 1998).

Potential Adverse Effects of Isoflavones

Dietary intake of isoflavones can elicit a variety of biological effects (see review

by Setchell 1998). Plants with estrogenic activity have been used for centuries to increase or decrease fertility (see review by Molteni, Brizio-Molteni, and Persky 1995). Compounds derived from plants, notably isoflavones, affect the reproductive tract and the reproductive cycle of animals (see review by Molteni, Brizio-Molteni, and Persky 1995). Early findings have shown that phytoestrogens were the cause of an infertility syndrome known as *Clover Disease* (Bennetts, Underwood, and Shier 1946). Molteni and coworkers (1995) noted that sheep that have ingested *equol precursors* are susceptible to extreme reproductive disorders; including mammary gland abnormalities, uterine inertia, cessation of uterine contractions during labor, and uterine prolapse, development of cysts in the uterus lining.

Animal studies have confirmed the original findings by Bennetts and coworkers (1946) that phytoestrogens may cause sexual developmental disorders. Phytoestrogens have been shown to compete effectively with estrogen for binding sites in sheep uterine receptors *in vitro* (Shutt and Cox 1972). It has been suggested that phytoestrogens may interfere with the release of gonadotrophins from the pituitary gland, hormones that support and stimulate the function of sex glands (see review by Molteni, Brizio-Molteni, and Persky 1995). By competing with estrogens for binding with estrogen receptors in the hypothalamus, phytoestrogens may cause *interruption of the feedback-regulating system of the hypothalamus-pituitary-gonadal axis* (see review by Molteni, Brizio-Molteni, and Persky 1995). This finding suggests that phytoestrogen intake may be the cause of infertility in sheep (see review by Molteni, Brizio-Molteni, and Persky 1995) by suppressing normal reproductive functions (Whitten et al. 1995).

Whitten et al. (1995) found that high concentrations of phytoestrogens in young female and male rats caused altered sexual development. Young female rats fed high

levels of phytoestrogens developed *premature anovulatory syndrome*, which is characterized by the suppression of ovulation (Whitten et al. 1995). High levels of phytoestrogens in male rat pups prevented the *masculinization of sexual behavior and defeminization of gonadotropin function* (Whitten et al. 1995).

Some studies show that there may be potential adverse effects upon infants fed soy-based formulas (Irvine, Fitzpatrick, and Alexander 1998 and Irvine et al. 1995). Recently, researchers have expressed concern about exposure of infants to soy-based formulas at critical stages of development, that such exposure could alter the development of the reproductive system in infants (Setchell and Cassidy 1999 and Irvine, Fitzpatrick, and Alexander 1998). Irvine, Fitzpatrick, and coworkers (1998) stated that the amount of isoflavone ingested by infants fed soy-based formulas is more than four times the amount that can *alter reproductive hormone secretion in cyclic women.*

Irvine, Shand and coworkers (1998) found that infants fed soy-based formulas were able to digest, absorb, and excrete isoflavones as efficiently as adults fed soy products. Researchers measured the isoflavone concentration in soy-based formulas, formulas based on cow or goat milk, and breast milk from vegetarian or omnivorous women (Irvine, Shand et al. 1998). No isoflavones were found in the cow, goat, or breast milk (Irvine, Shand et al. 1998). Furthermore, no isoflavones were detected in the urine of infants fed the dairy-based formulas (Irvine, Shand et al. 1998). Isoflavones were detected in the urine of infants fed soy-based formulas (Irvine, Shand et al. 1998).

Dietary Fiber

Dietary fiber is nondigestable material (Whistler and BeMiller 1997) found in the following plant derived food sources: fruits and vegetables, cereals, and legumes

(Mongeau 1993). It consists of water-insoluble plant components, primarily cellulose and lignins as well as water-soluble plant components, non-starch polysaccharides (Whistler and BeMiller 1997). These plant components cannot be hydrolyzed by endogenous enzymes of the human upper gastrointestinal tract (Schaller 1978), found in secretions from mouth, stomach, gall bladder, exocrine pancreas, and small intestine (Mongeau 1993). The principal constituents of dietary fiber are shown in Table 1.

Table 1. Principal macromolecular constituents of dietary fiber

Constituents	Fruits and vegetables	Cereals	Legumes
Polysaccharide			
Hemicelluloses			
Xyloglucans	X		X
Glucuronoxylans	X		
Arabinoxylans		X	
Glucuronoarabinoxylans		X	
Galactomannans			X
Cellulose	X	X	X
ß-D-Glucans		X	
Pectic substances	X	X	X
Others			
Lignin	X	X	
Phenolic esters		X	
Protein		X	
Glycoproteins	X		X

(Mongeau 1993).

The American Dietetic Association recommends that adults consume from 20 to 35 g of dietary fiber per day (Whitney and Rolfes 1999).

Dietary fiber from most sources is highly fermentable. Most soluble fiber and variable amounts of insoluble fiber are degraded by bacteria in the large intestine by fermentation (Gordon and Pellett 1992). When bacteria in the intestinal tract ferment fibers, water, gas, and short-chain fatty acids (SCFA) are produced (Whitney and Rolfes 1999). Short-chain fatty acids *are either utilized by the mucosal cells of the large intestine or transported to the liver* (Gordon and Pellett 1992). Short-chain fatty acids (SCFA) metabolized by colonic mucosal cells, include acetate, butyrate, and propionate; butyrate is the preferred fuel for these cells (Slavin et al. 1999).

Dietary fiber and short-chain fatty acid production are potential mechanisms for lowering blood cholesterol and reducing the risk of cancer (Slavin et al. 1999). SCFA, in particular butyric acid, are thought to act as *regulators of cell cycling and metabolism* (Gordon and Pellett 1992). The absorption and transfer of SCFA to the liver have been suggested as a regulator of cholesterol biosynthesis (Gordon and Pellett 1992). Dietary fiber lowers blood cholesterol because fibers entrap bile acids in the large intestine, thereby removing the bile from the body via the feces (Whitney and Rolfes 1999). Cholesterol is required to make bile; *the excretion of bile effectively reduces blood cholesterol* (Whitney and Rolfes 1999).

Dietary fibers are considered bulking agents that promote the normal physiological function of the gastrointestinal tract (Whistler and BeMiller 1997). A bulking agent increases intestinal and fecal bulk, thereby shortening the intestinal transit time (Whistler and BeMiller 1997). A decreased intestinal transit time decreases the period of time that *fecal mutagens* can *interact with the intestinal epithelium* (Slavin et al. 1999). Increased fecal bulk reduces the time available for intestinal ß-glucuronidase activity and for steroid absorption and participation in the bile acid enterohepatic

circulation (see review by Slavin 1994). Thus, fiber-rich foods are thought to decrease blood cholesterol levels and reduce the risk of colon cancer and heart disease (Whistler and BeMiller 1997). Slavin et al. (1999) concluded that *soluble fiber is associated with cholesterol-lowering effects and improved glucose response, whereas insoluble fiber is associated with improved laxation.*

Insoluble fiber. Cellulose is the principal component of the cell wall of plant cells and is the most abundant organic compound on Earth (Nussinovitch 1997). It is an insoluble, linear polymer of ß-D-glucose monomers joined by ß (1-4) glycosidic bonds (See Figure 7) (Nussinovitch 1997).

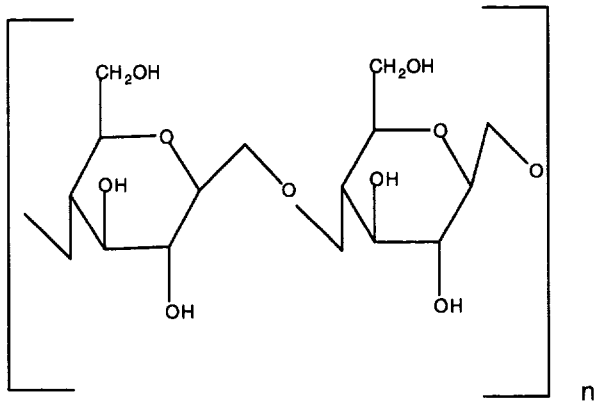


Figure 7. Chemical structure of repeating ß-D-glucose units in cellulose.

Cellulose molecules form polycrystalline regions, in which the molecular chains in the crystalline regions are held together by numerous hydrogen bonds (Whistler and BeMiller 1997). Cellulose is insoluble in water due to intra- and intermolecular hydrogen-bonded crystalline regions (Nussinovitch 1997). Each hydrogen-bonded crystalline region prevents internal hydration within that region (Lewis 1978). In general, linear polysaccharides are insoluble in water, whereas branched polysaccharides favor improved solubility (Lewis 1978).

Soluble fiber. Locust bean gum (LBG), also known as carob gum, is the ground endosperm of locust bean seeds (Whistler and BeMiller 1997). Locust bean seeds are obtained from the pod of the locust tree that is grown near the Mediterranean Sea (Whistler and BeMiller 1997). Locust bean gum is a soluble polysaccharide of ß (1-4) linked D-mannan units, *a linear homoglycan of ß-D-mannopyranosyl units*, with repeating α-D-galactose units at C-6 of mannose (See Figure 8) (Nussinovitch 1997).

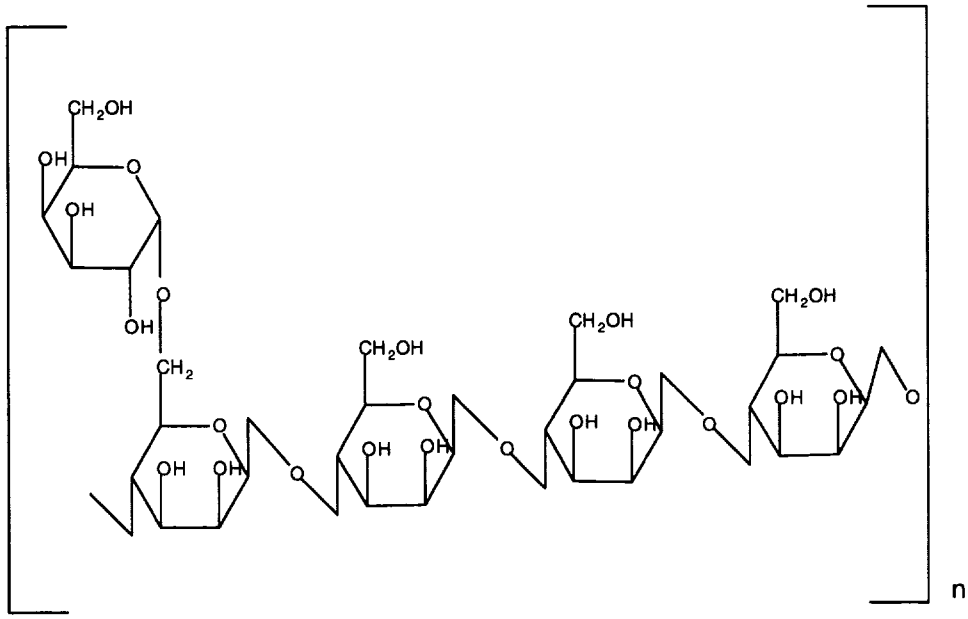


Figure 8. Chemical structure of the repeating units in locust bean gum.

Dietary Fiber/Isoflavone Interaction

Studies have shown that dietary fiber binds phytoestrogens (Tew et al. 1996) and human estrogens (Rose et al. 1991, Whitten and Shultz 1988, and Shultz and Howie 1986). When dietary fiber binds to phytoestrogens or human estrogens, the estrogen/estrogen-like compounds are more readily excreted from the body causing decreased bioavailability of the compounds.

Dietary fiber inhibits the absorption of phytoestrogens (Tew et al. 1996). To determine the effect of dietary fiber on the bioavailability of isoflavones, Tew et al. (1996) randomly assigned either of two diets to women; a control diet containing 15 g of dietary fiber or a wheat fiber-supplemented diet containing 40 g of dietary fiber. Women on each diet consumed 0.9 mg isoflavones/kg body weight per day. They found that total urinary excretion of genistein was lower upon consumption of a high fiber diet compared to the control diet. In contrast, urinary excretion of daidzein was not significantly affected by fiber intake (Tew et al. 1996). Both plasma daidzein and genistein concentrations were significantly lower in the women who consumed the fiber-rich diet than the control diet. The authors suggest that the lower urinary excretion of isoflavones detected after consumption of the fiber-rich diet may be due to a decrease in the bioavailability of the isoflavones resultant from their being bound to dietary fiber thus hindering absorption of the isoflavones (Tew et al. 1996). Furthermore, the authors suggest that urinary genistein excretion, not urinary daidzein excretion, was affected by dietary fiber most likely because *genistein is more hydrophobic than daidzein* (Tew et al. 1996). The hydrophobic binding preference of dietary fibers for human estrogens was observed by Shultz and Howie (1986).

Studies have shown that human estrogens bind dietary fiber (Shultz and Howie 1986). Shultz and Howie (1986) investigated the *in vitro* binding of conjugated steroid and unconjugated steroid hormones by natural (ex. brans or oat hulls) and purified (ex. cellulose or lignin) fiber. They found that the conjugated and unconjugated steroid hormones were bound, to varying degrees, by each type of fiber. Binding of the conjugated steroid hormone to each type of fiber was less than the unconjugated steroid hormones (Shultz and Howie 1986). Cellulose was shown to have the least binding

capacity for steroids of any of the fibers studied (Shultz and Howie 1986). Shultz and Howie (1986) stated that *the results support the hydrophobic nature of adsorption* between steroid hormones and dietary fiber. In addition, Shultz and Howie (1986) indicated that dietary fiber may influence the metabolism of steroid hormones by affecting the enterohepatic circulation of these hormones.

Whitten and Shultz (1988) found that steroid hormones bind water-insoluble dietary fibers. This *in vitro* study examined the binding capabilities of steroid hormones to water-insoluble fiber fractions of composite food samples prepared for and consumed by omnivorous and vegetarian subjects (Whitten and Shultz 1988). Estrone and estradiol-17ß bound more strongly to water-insoluble fiber than testosterone (Whitten and Shultz 1988). The authors stated that *binding of steroid hormones by dietary water-insoluble fiber is related to the actual fiber content of the diet* (Whitten and Shultz 1988).

Rose et al. (1991) found that consumption of a high-fiber diet lowers blood estrogen levels in premenopausal women. They studied premenopausal women who increased their dietary fiber intake by approximately 15 to 30 grams per day by supplementation of wheat, oat, or corn bran (Rose et al. 1991). The women who consumed the wheat-bran-supplemented diet showed a significant reduction in blood estrogen levels, whereas the blood estrogen levels were unaffected in women who consumed the oat or corn-bran-supplemented diet. No change in sex-hormone-binding globulin levels in blood of women consuming the wheat-bran-supplemented diet were observed despite the reduction in blood estrogen levels (Rose et al. 1991).

Dietary Fiber/Iron Interaction

Components of fiber have been found to bind iron *in vitro* (Fernandez and Phillips

1982). The effects of dietary fiber on iron absorption may reduce the bioavailability of dietary iron (Fernandez and Phillips 1982, Reinhold, Garcia, and Garzon 1981, and Ismail-Beigi, Faraji, and Reinhold 1977). Dietary fibers, including cellulose, psyllium mucilage, pectin, and lignin, were incubated with ferrous sulfate ($FeSO_4 \cdot 7\ H_2O$) in solution at various pH's and concentrations to determine the degree of binding by different fibers (Fernandez and Phillips 1982). Fernandez and Phillips (1982) found that pH influenced the degree of iron binding by different fibers. An increase in iron binding was seen at higher pH levels and a wider pH range with psyllium mucilage and lignins than cellulose and pectin (Fernandez and Phillips 1982). In addition, low concentrations of certain fibers were found to bind iron; lignins and psyllium mucilage bound iron at concentrations less than 1 mg/mL, whereas cellulose and pectin were much less effective, even at greater concentrations than achieved under physiological conditions (Fernandez and Phillips 1982).

Reinhold and coworkers (1981) found that iron was bound by fiber of wheat and maize. Reinhold et al. (1981) found that iron's ability to bind dietary fiber is dependent on several factors: iron concentration, pH, amount of fiber, and the amount of inhibitors of binding present or absent in the system. Ismail-Beigi, Faraji, and Reinhold (1977) also studied binding capability of iron to fiber components and found that iron binding by fiber was highly pH-dependent. These studies have shown that binding of dietary iron by fiber components may explain why decreased bioavailability of iron is seen in selective populations: rural areas of Iran and other Middle Eastern Countries (Ismail-Beigi, Faraji, and Reinhold 1977).

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a very powerful technique. In chromatographic analysis, the separation of a mixture into individual components is achieved by using a mobile phase (the solvent) with the stationary phase (the column packing). The detector is instrumental in the quantitative determination and/or qualitative identification of organic compounds. The components of a mixture are separated in chromatography due to their physical and chemical interactions with the mobile and stationary phase (Henschen et al. 1985). The sample and the mobile phase move through the column where separation of the components takes place. The *eluate*, the substance obtained from elution in chromatography, moves from the column to the detector which records both the retention time of each compound and the response from the detector. Five main parts of the chromatograph will be outlined: solvent reservoir, high pressure pumps, sample injection system, column, and detector and recording unit.

Solvent reservoir. The solvent composition used in this HPLC research was HPLC grade methanol/1mM of ammonium acetate (6:4, v/v). Before the solvent was used in chromatographic analysis, it was degassed to remove gas bubbles that may interfere with the pump system. Degassing was achieved by filtering the mobile phase with a milli-pore system which operates in conjunction with a vacuum pump.

High pressure pumps. The Water's Associates chromatography pump was a piston-type constant volume pump. The pump operates when a forward movement of the piston pushes solvent through a non-return valve on the column side, and the backward stroke draws more solvent from the solvent reservoir (Hamilton and Sewell 1982). The pump is instrumental in creating pressure to move the solvent through the column towards the detector.

Sample injection system. The sample to be analyzed is injected via an autosampler. The volume of the sample injected is determined by the experimenter. The sample is introduced into the mobile phase by a syringe, thereby moving the sample towards the column where partitioning occurs.

Column. The stationary phase in the μ-Bondapak C_{18} column consists of a C_{18} compound bonded to silica. The packing material in the column is a non-polar compound. The *analyte*, the element, compound, or chemical species that is tested, is attracted to the solid surface within the stationary phase, resulting in polarity attraction. The partitioning of the components within a mixture occur in the column. The compounds *elute*, pass through and exit from the column, in the order of molecular weight and solubility interactions with the packing material. The time required for the components to pass through the column is called the *retention time.* The retention time is measured from the onset of injection, to the point where the sample reaches the detector.

Detector and recording unit. A photo-diode array detector is used for optical spectrometric measurements (Strobel and Heineman 1989). The detector has the ability to monitor specific wavelengths or scan all wavelengths within its range. The radiation source used in the UV detector was a deuterium lamp. The detector senses the separated components of the sample and sends the electric signal to the amplifier. The chart recorder then records the detected voltage. Qualitative identification of a compound is determined by the retention time. The quantitative determination of the compound is determined by the area under each peak. The peak is quantitatively proportional to the concentration of compound present.

METHODOLOGY

The purpose of this research was to investigate quantitative differences in binding of the phytoestrogen, daidzein, by a soluble fiber (locust bean gum) and an insoluble fiber (cellulose) both in the presence and absence of iron. Included in this chapter are descriptions of the materials, sample preparation, instrumentation, and statistical analysis used in this phytoestrogen study.

Materials

Daidzein (7, 4'-dihydroxyisoflavone), minimum purity 98%, and reagent grade ammonium acetate were obtained from Sigma Chemical Company (St. Louis, MO). The soluble dietary fiber, locust bean gum (LBG), was a gift from TIC Gums, Inc. (Belcamp, MD). The insoluble dietary fiber, cellulose (Solka Floc™), was obtained from James River Corporation (Richmond, VA). The mineral, reagent grade ferrous sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$) and HPLC grade methanol were obtained from Fisher Scientific, Fairlawn, NJ. Sodium hydroxide and hydrochloric acid, both reagent grade, were purchased from Spectrum Chemical Mfg. Corp, Gardena, CA.

Standard Solutions and Calibration Curves

Daidzein stock solutions were prepared by dissolving the compound first in methanol followed by addition of Milli-Q water to produce a concentrated solution. The stock solution of the standards were diluted to concentrations of 1.00, 2.00, 4.00, 8.00,

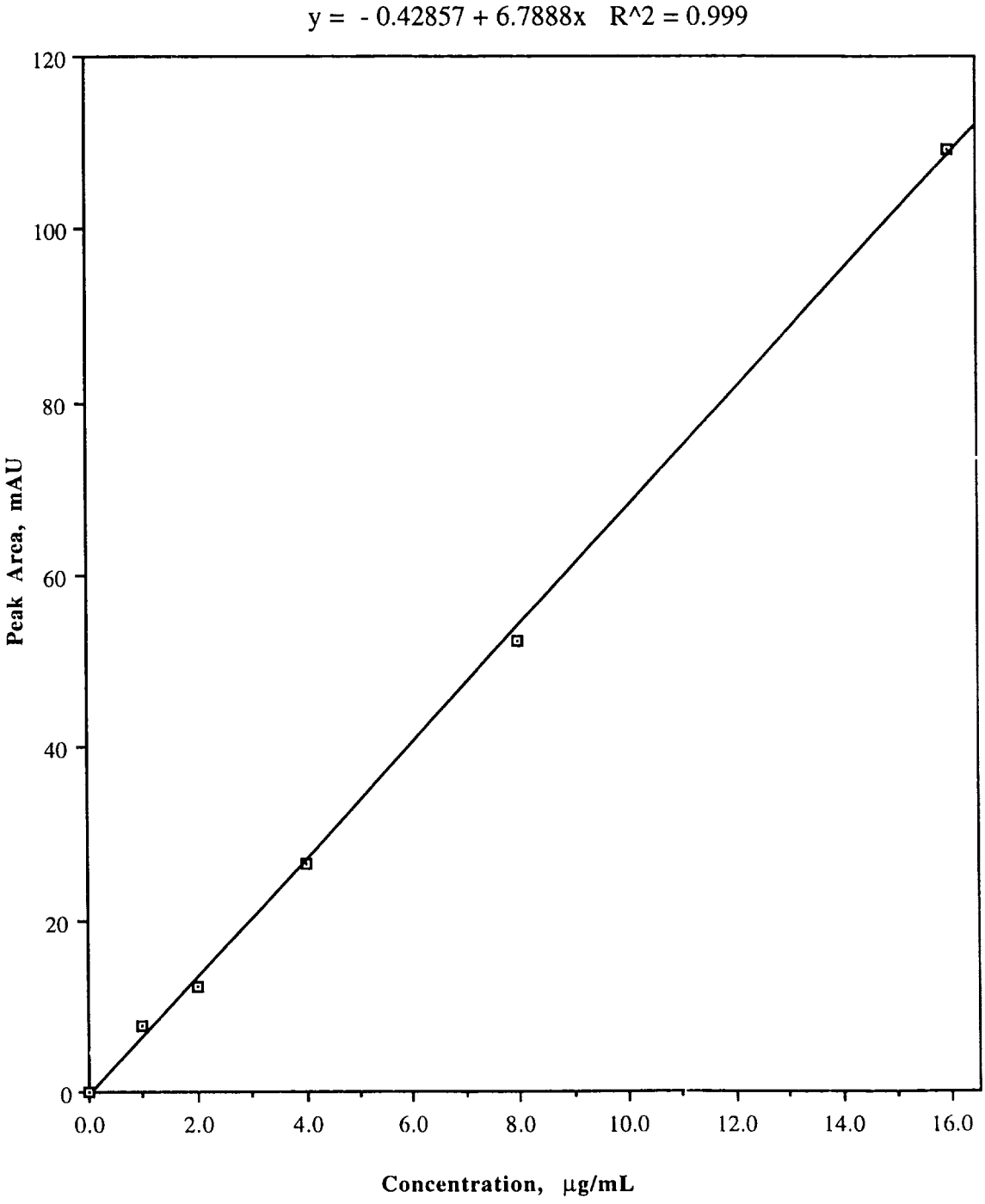
and 16.0 μg/mL (See Table 2). Calibration curves created from these standards demonstrated a high degree of linearity ($r > 0.999$) upon plotting standard concentration versus peak area (mAU) obtained from the HPLC analyses with 15 μL injections. Each concentration was analyzed by triplicate injections.

Table 2. Standard solutions for daidzein analysis

Standard Solution (μg/mL)	Amount of Stock Solution (50μg/mL) diluted to 25 mL (mL)
0.00	0.00
1.00	0.50
2.00	1.00
4.00	2.00
8.00	4.00
16.0	8.00

The linearity of each standard solution was determined by preparing a range of standards. The calibration curve of daidzein was obtained by using Cricket Graph Version 1.3.1 (Cricket Software, Inc., Malvern, PA). Refer to Figure 9 for the calibration curve for daidzein standards.

Figure 9. Calibration curve for daidzein standards.



Procedure

The samples analyzed in this study contained one or more of the following compounds in each system. Each phytoestrogen system is outlined in Table 3.

Table 3. Composition of phytoestrogen systems studied

Sample System	Fiber	Mineral
Phytoestrogen	—	—
Phytoestrogen and mineral	—	+
Fiber	LBG	—
Fiber	Cellulose	—
Mineral	—	+
Fiber and mineral	LBG	+
Fiber and mineral	Cellulose	+
Phytoestrogen and fiber	LBG	—
Phytoestrogen and fiber	Cellulose	—
Phytoestrogen, fiber, and mineral	LBG	+
Phytoestrogen, fiber, and mineral	Cellulose	+

LBG = Locust bean gum
Mineral = Fe
+ = was added to the system
— = was not added to the system

Samples were prepared by the following procedure: The appropriate fiber (250 mg) was added to 32.0 mL of Milli-Q water and hydrated on a stir/hotplate for 30 minutes at 37° C. Following the hydration step, selected samples received ferrous sulfate heptahydrate solution, 1.12 mg/mL, to produce a fiber-mineral mix. Then 1.7 mg of daidzein was added to the hydrated fiber or hydrated fiber-mineral mix. The samples were adjusted to a pH of 2.0 by the addition of 1.0, 0.1, 0.05, and 0.0125 M hydrochloric acid (HCl) and allowed to incubate for 30 minutes at 37° C with constant stirring. Subsequent to that, the samples were adjusted to a pH of 6.5 by the addition of 1.0, 0.1, 0.05, 0.0125 M sodium hydroxide (NaOH) and incubated with stirring for an additional 30 minutes at 37° C.

The samples were diluted to 50.0 mL in a volumetric flask with Milli-Q water, transferred to a centrifuge tube that was centrifuged for 30 minutes at 1300 x *g*. After centrifugation, the supernatant was removed, filtered through a 0.45 μm Whatman Puradisc™, and analyzed for unbound phytoestrogens using high performance liquid chromatography (HPLC). Each phytoestrogen system was analyzed in triplicate.

Instrumentation

HPLC analysis was performed on a Waters Associates chromatograph equipped with a Model WISP 710B autosampler (Waters Associates, Milford, MA), a Model 6000A chromatography pump (Waters Associates, Milford, MA), and a Model 1040A photo-diode array detector (Hewlett-Packard, Palo Alto, CA). Compounds were separated by a reversed-phase μ-Bondapak C_{18} (30cm x 3.9mm, Waters Associates) column. Compounds were eluted from the column at a flowrate of 1.0 mL/min utilizing the following isocratic solvent system: methanol/1mM ammonium acetate (6:4, v/v)

(Wang et al. 1990). Analytes were monitored by the photo-diode array detector at a wavelength of 254 nm.

Statistical Analysis

A statistical analysis software program, SPSS for *Windows* Version 6.0 (SPSS Inc., Chicago, IL), was used to perform the Independent Sample *t*-test to determine the degree of significance between the concentration of unbound daidzein in the presence of fiber or fiber and mineral complex. In addition, the *t*-test was used to determine the degree of significance between the concentration of unbound daidzein in the *insoluble* fiber and mineral complex in comparison to the *soluble* fiber and mineral complex. The statistical tests were considered to be significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

This study was conducted to determine the quantitative differences in the concentration of unbound daidzein between samples containing soluble fiber (locust bean gum) and insoluble fiber (cellulose) both in the presence and absence of iron. The qualitative determination of daidzein by HPLC was based on the comparison of the retention times of the standard solutions with sample peaks. The data collected on daidzein analysis is reported in this chapter.

HPLC Analysis of Daidzein in Samples

Daidzein was eluted from a μ-Bondapak C_{18} column by an isocratic solvent system of methanol/lmM ammonium acetate (6:4, v/v) at a flowrate of 1.0 mL/minute. The compound isolated from experimental samples produced a symmetric peak having a retention time of 6.4 ± 0.2 minutes that was almost identical to the retention time of the standard solutions of daidzein.

Determination of Unbound Daidzein in Samples

Samples (n=5) for each experimental condition were analyzed in triplicate by HPLC for unbound daidzein content. The unbound daidzein represents the pool of daidzein potentially available for absorption. The equation for linearity ($y = -0.42857 + 6.7888x$) derived from the standard curve for daidzein concentrations was used to calculate the concentration of daidzein in each experimental system.

When daidzein was added to samples containing dietary fiber, the concentration of unbound daidzein was different from the control samples that contained no fiber, see Table 4. There was a significant increase in the concentration of unbound daidzein in the presence of locust bean gum (62.2 μg/mL ± 3.17) ($p < 0.001$) and a significant increase in the concentration of unbound daidzein in the presence of cellulose (27.8 μg/mL ± 0.374) ($p < 0.001$) compared to the concentration of daidzein in the control sample (17.4 μg/mL ± 0.697). These results suggest that both insoluble and soluble fiber take up more water than daidzein from the time that daidzein was added causing the concentration of unbound daidzein to increase in the unbound water with each fiber sample.

Table 4. Effect of cellulose and locust bean gum on the concentration of unbound daidzein with or without iron

Sample System	Concentration of Unbound Daidzein (± SEM)
Control	**$17.4^{a} \pm 0.697$**
Locust Bean Gum	**$62.2^{b} \pm 3.17$**
Locust Bean Gum and Iron	**$63.7^{b} \pm 3.86$**
Cellulose	**$27.8^{c} \pm 0.374$**
Cellulose and Iron	**$31.3^{c} \pm 1.64$**

a,b,c means with the same superscript are not significantly different at $p \leq 0.05$.
SEM = Standard Error Mean

There were significant differences (Table 4) between the concentration of unbound daidzein in samples containing the soluble fiber, locust bean gum (62.2 μg/mL ± 3.17) compared to the concentration of unbound daidzein in samples containing the insoluble fiber, cellulose (27.8 μg/mL ± 0.374) ($p < 0.001$). In the presence of iron, see Table 4, a similar relationship was observed ($p < 0.001$). The daidzein concentration in the locust bean gum, daidzein, and iron samples were 63.7 μg/mL ± 3.86 compared to that obtained from the cellulose, daidzein, and iron samples, 31.3 μg/mL ± 1.64.

Iron did not significantly affect the concentration of unbound daidzein in the presence of either fiber. There were no significant differences observed in the concentration of unbound daidzein in solution between the sample with *soluble* fiber and iron (62.2 μg/mL ± 3.17) and the sample with *soluble* fiber alone (63.7 μg/mL ± 3.86), see Table 4 ($p < 0.630$). A similar relationship was observed ($p < 0.092$) with *insoluble* fiber and iron (31.3 μg/mL ± 1.64) and *insoluble* fiber alone (27.8 μg/mL ± 0.374), see Table 4.

Since dietary fibers have been reported to bind or entrap phytoestrogens (Tew et al. 1996), this study was designed to investigate the quantitative differences in binding of the phytoestrogen, daidzein, by insoluble and soluble fibers in the presence and absence of iron supplementation. When daidzein was added to samples of dietary fiber, the concentration of the unbound daidzein increased significantly compared to the control sample containing no fiber (see Table 4). The difference in the concentration of unbound daidzein between the control sample and the samples containing fiber may be attributed in part to differences in the extent of hydration of each fiber at the time

daidzein was added. It is possible that the total amount of water absorbed by the two fibers was different after the addition of daidzein. Since the concentration of unbound daidzein was greater in the supernatant from locust bean gum samples than in the supernatant from cellulose samples, the data suggest that the soluble fiber, locust bean gum, absorbed more water after addition of daidzein than an equal weight of the insoluble fiber, cellulose. Some support for this idea was obtained during sample preparation.

Locust bean gum suspensions were more viscous than cellulose suspensions. It was considerably more difficult to filter locust bean gum suspensions than cellulose suspensions making it harder to obtain filtrate from the locust bean gum suspensions than cellulose suspensions. When the supernatant from locust bean gum suspensions was micron-filtered, considerable pressure was required to force the sample through the filtration system. In contrast, the insoluble fiber suspension was much less viscous and was easily filtered with application of much less pressure to the sample. If proportionately more water bound to fiber, less water would be available for daidzein in solution, causing the concentration of daidzein in the unbound water of the supernatant to be higher.

If the total amount of water bound to equal masses of these fibers is essentially the same, the data would suggest, instead that a greater amount of daidzein is bound to cellulose than to locust bean gum. It has been reported that dietary fibers bind or entrap phytoestrogens (Tew et al. 1996). Such a difference might be attributed in part to the presence and availability of hydrophobic regions within the fibers. The structure of daidzein suggests that it has significant hydrophobic character. If the fibers are entrapping and/or binding to phytoestrogens or human estrogens within polycrystalline

regions (Tew et al. 1996, Whitten and Shultz 1988, and Shultz and Howie 1986), then daidzein may potentially be removed from the system to a greater extent by the *insoluble* fibers than by the *soluble* fibers.

These two possible explanations are not mutually exclusive. It is possible that daidzein is bound to a greater extent by one fiber than another and also possible for the two fibers to differ in the amount of water bound per gram of fiber. This study does not distinguish between these two possibilities.

The present study showed that mineral supplementation did not significantly affect the ability of the fibers to bind daidzein. No data were found in the literature on the effect of mineral supplementation on the binding of phytoestrogens or human estrogens to fiber. Fernandez and Phillips (1982) reported, however, that components of fiber do remove and bind iron from solution. It is possible that iron did not affect the amounts of daidzein bound to fibers to any significant degree, due to different kinds of binding sites necessary for each compound. The fiber-iron interaction could occur as an ion-dipole interaction forming an iron-fiber complex, whereas the fiber-daidzein interaction would likely be at least partially hydrophobic in nature. It is possible that iron and daidzein do not compete for the same binding sites on fiber due to the nature of their interaction. Ismail-Beigi and coworkers (1977) indicated that *cellulose binds metals and that an exchange of metal ions for protons occurs*. This suggests an ion-ion binding of iron to fiber (Ismail-Beigi, Faraji, and Reinhold 1977).

This study has shown that insoluble and soluble fibers may differ in their ability to bind daidzein. Soluble fiber appeared to bind considerably less daidzein than insoluble fiber. If indeed, insoluble fibers bind significant amounts of daidzein, then the total amount of daidzein that is available for absorption may be decreased. The insoluble

fiber, cellulose, is able to form polycrystalline regions within its structure, in which the hydroxyl groups of one glucose monomer hydrogen-bond to hydroxyl groups of another glucose monomer along the linear chain to create the polycrystalline regions (Whistler and BeMiller 1997). Researchers have suggested that fiber binds to isoflavones through hydrophobic interactions with the compound (Tew et al. 1996, Whitten and Shultz 1988, and Shultz and Howie 1986). Tew and coworkers (1996) found that insoluble wheat fiber reduced the absorption of the isoflavone, genistein, probably by its *bulking effect and hydrophobic binding* to the isoflavone compound. Rose et al. (1991) found that a wheat-bran-supplemented diet significantly lowered the blood estrogen levels in premenopausal women, which would support the idea that fiber binds to human estrogens resulting in a reduction in the bioavailability of the compound for absorption. These data suggest that diet may possibly modify the nutritional status of humans, possibly through the binding ability of dietary fiber.

SUMMARY AND CONCLUSION

The results of this study showed that equal masses of two dietary fibers, cellulose and locust bean gum, affect the amount of daidzein in solution. The insoluble dietary fiber, cellulose, appeared to bind a greater amount of daidzein than the soluble dietary fiber, locust bean gum, as measured by the concentration of daidzein remaining in the supernatant after exposure to equal masses of these fibers. High concentrations of unbound daidzein would facilitate its intestinal adsorption, whereas the presence of bound daidzein would decrease the total amount of daidzein available for absorption. The effect of mineral supplementation of the fiber-containing solutions did not significantly alter the concentration of unbound daidzein in solution. This result suggests that mineral supplementation may not affect the bioavailability of daidzein in solution.

Recommendations for Further Study

Based on the present study, recommendations for further study are as follows:

1. Study the effect of binding by different fibers to daidzein in solution. Since the insoluble fiber, cellulose, appeared to bind to daidzein more than the soluble fiber, locust bean gum, it would be interesting to find out if other soluble and insoluble fibers have similar binding capabilities, respectively.

2. Study the effect of binding by fiber to different phytoestrogens. Daidzein, a dihydroxyisoflavone, and genistein, a trihydroxyisoflavone, differ only by the addition of a hydroxyl group on the aromatic A-ring in the structure of genistein. It would be interesting to discover if there is a similar binding relationship between the two phytoestrogens.

3. Study the influence of cations; such as, Na, Ca, and/or Mg, on fiber's binding ability to phytoestrogens. The current study showed that mineral supplementation with iron had no significant affect on the binding ability of fibers to daidzein. It is possible that other minerals may affect binding.

4. Study the effect of binding by fiber to phytoestrogens by changing the current parameters, to include a study that more readily depicts the digestive process. The present study simulated the adjustment of pH and temperature of the sample system to represent the digestive process, not the environment expected in the human body.

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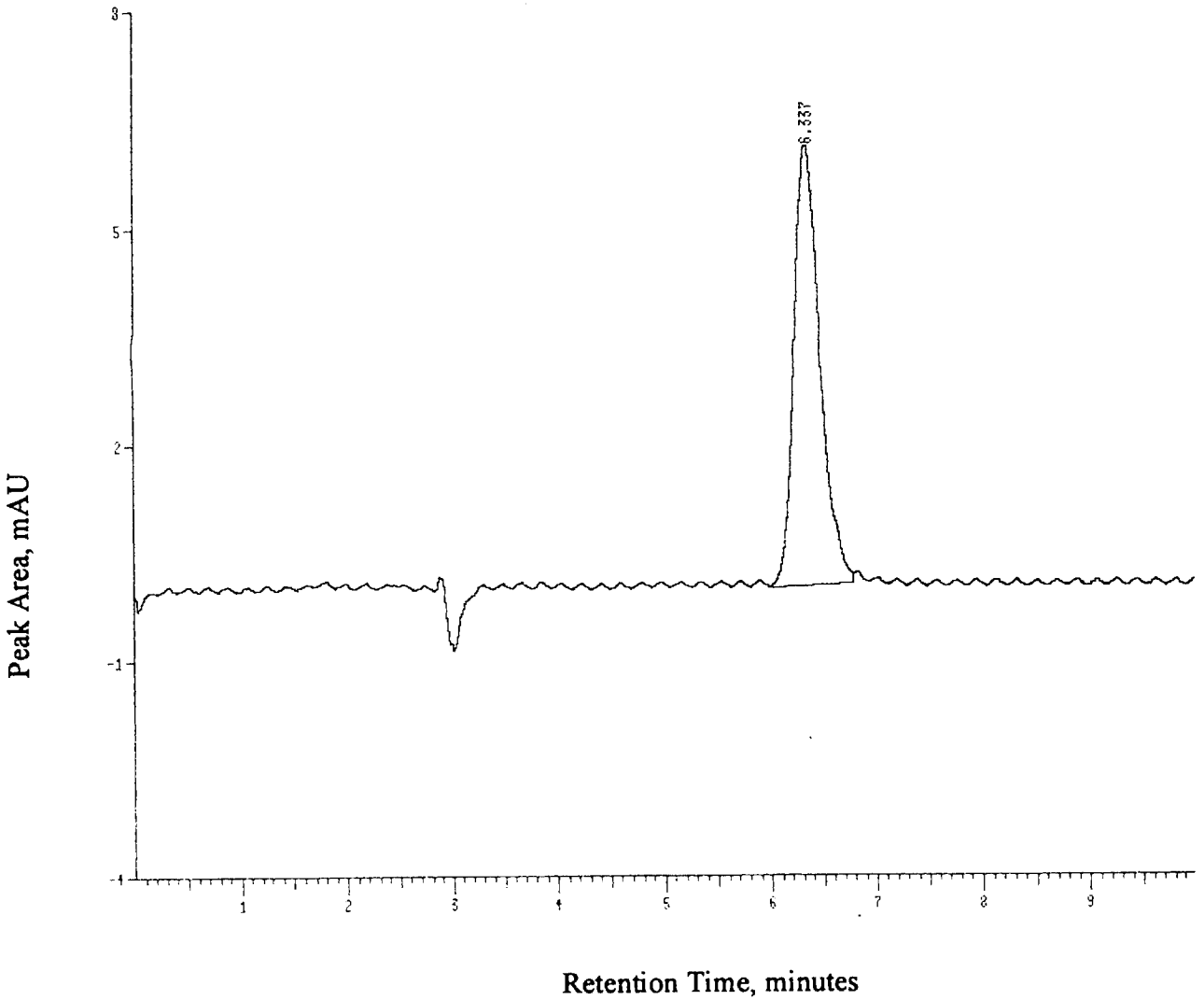

Figure 10. HPLC chromatogram of daidzein standard, 16.0 µg/mL.

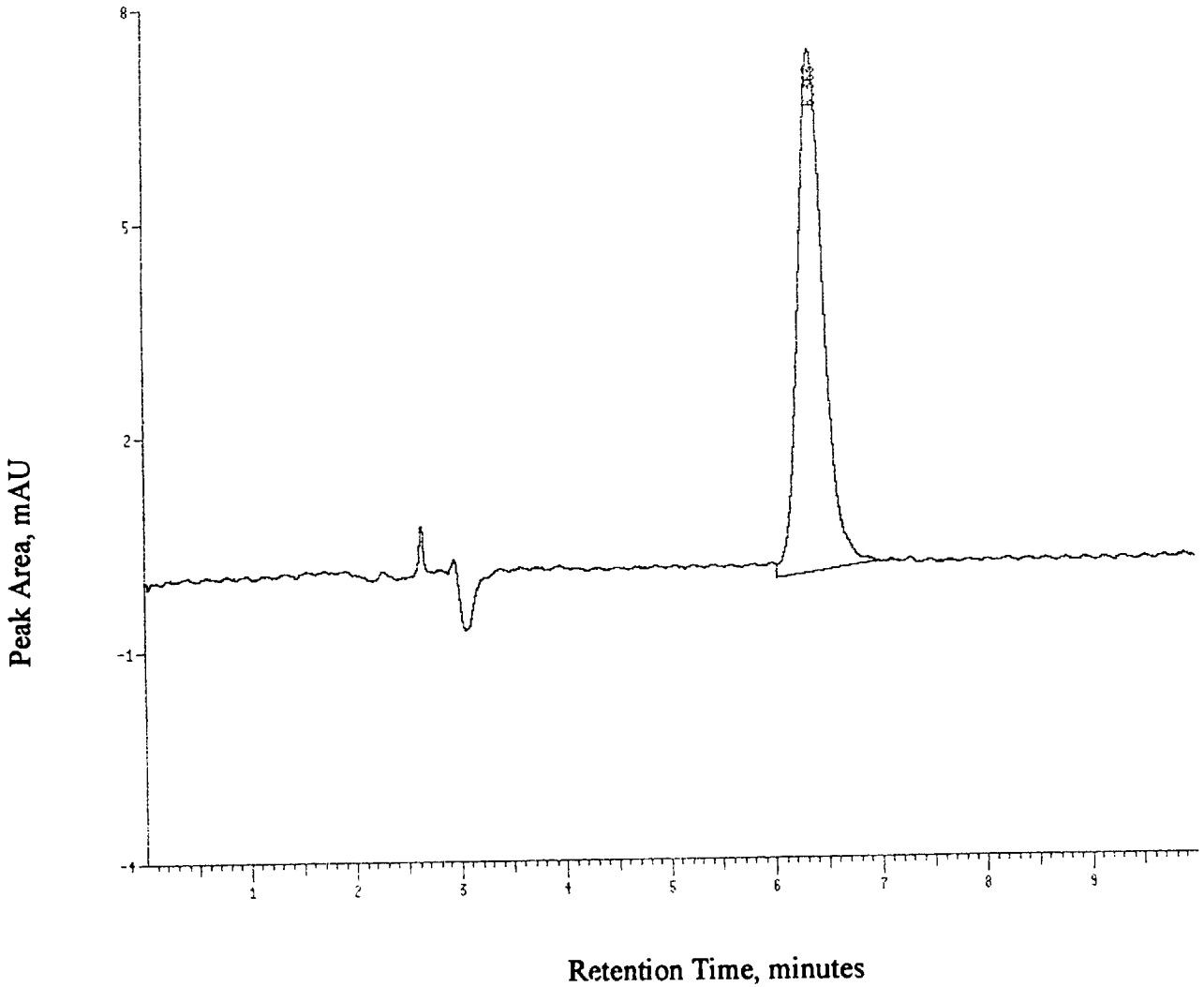

Figure 11. HPLC chromatogram of daidzein control sample, 34.0 µg/mL.

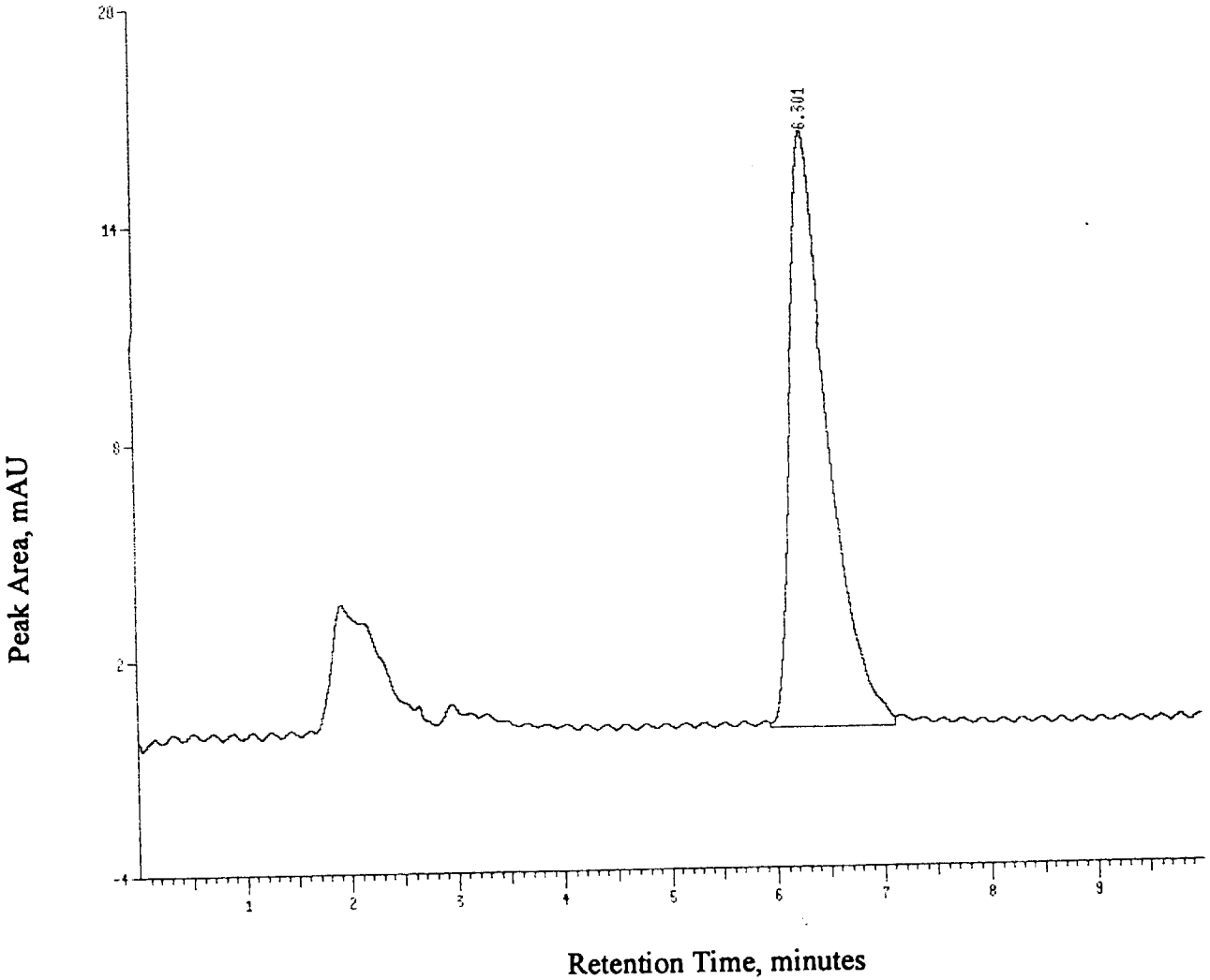

Figure 12. HPLC chromatogram of daidzein and locust bean gum sample.

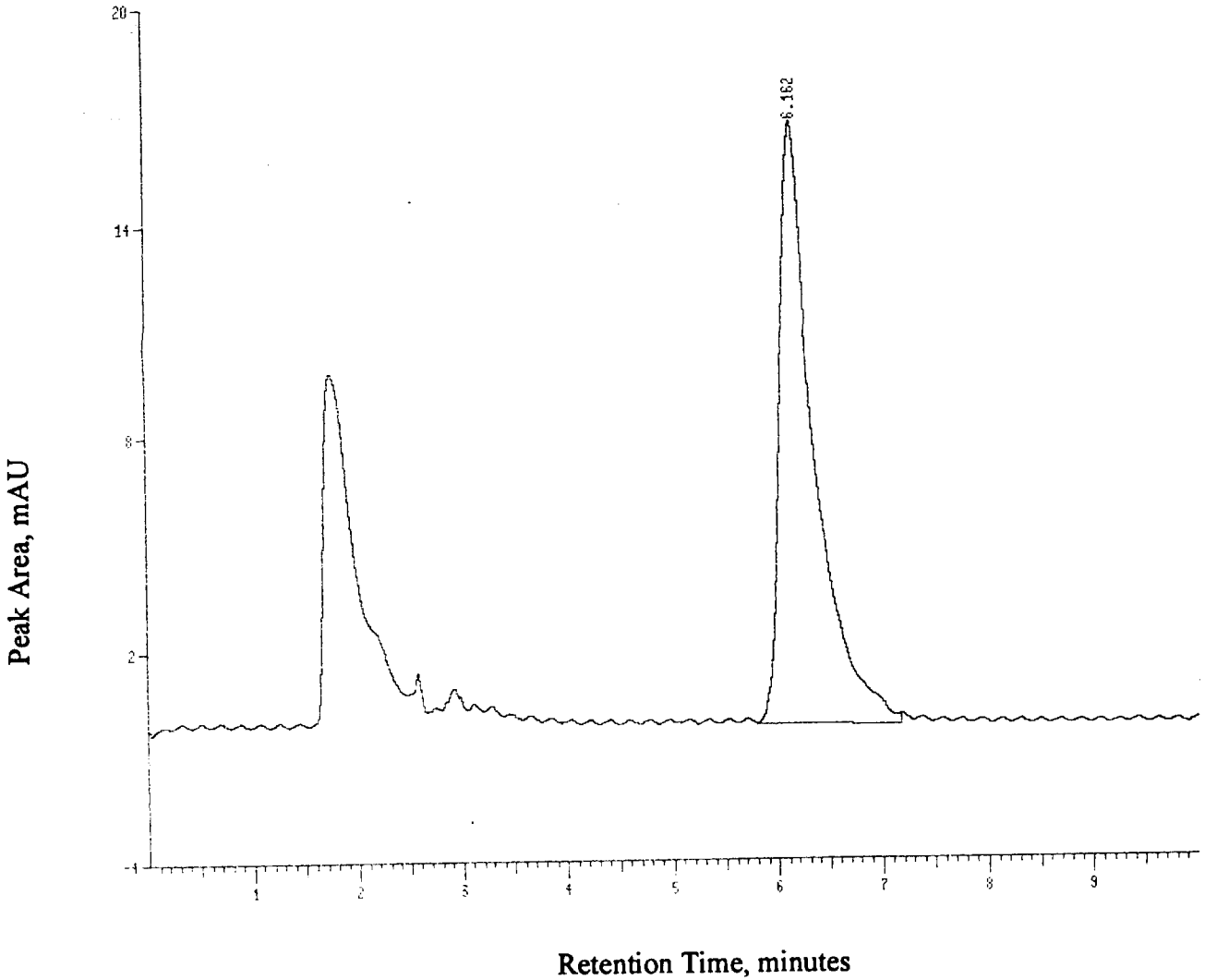

Figure 13. HPLC chromatogram of daidzein, locust bean gum, and iron sample.

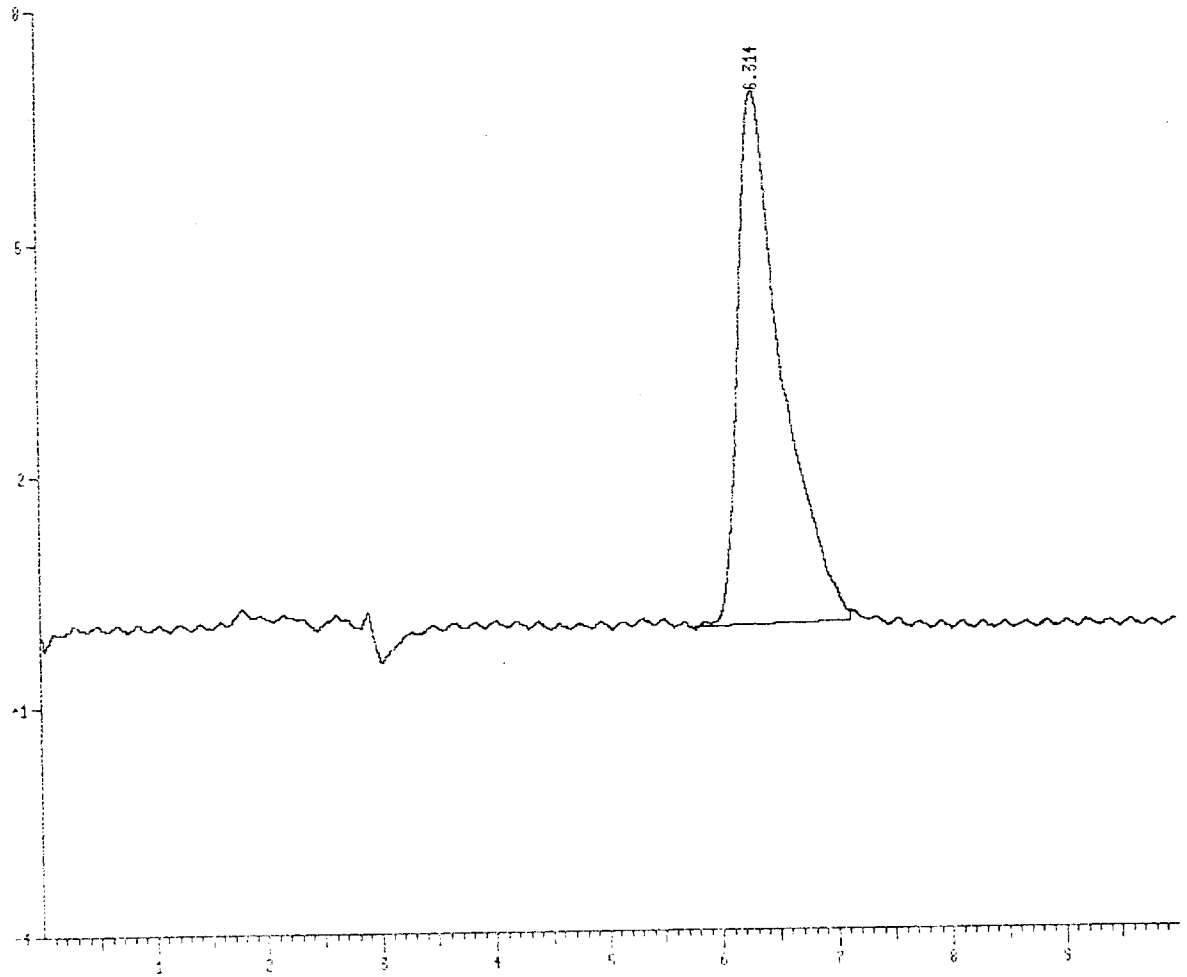

Figure 14. HPLC chromatogram of daidzein and cellulose sample.

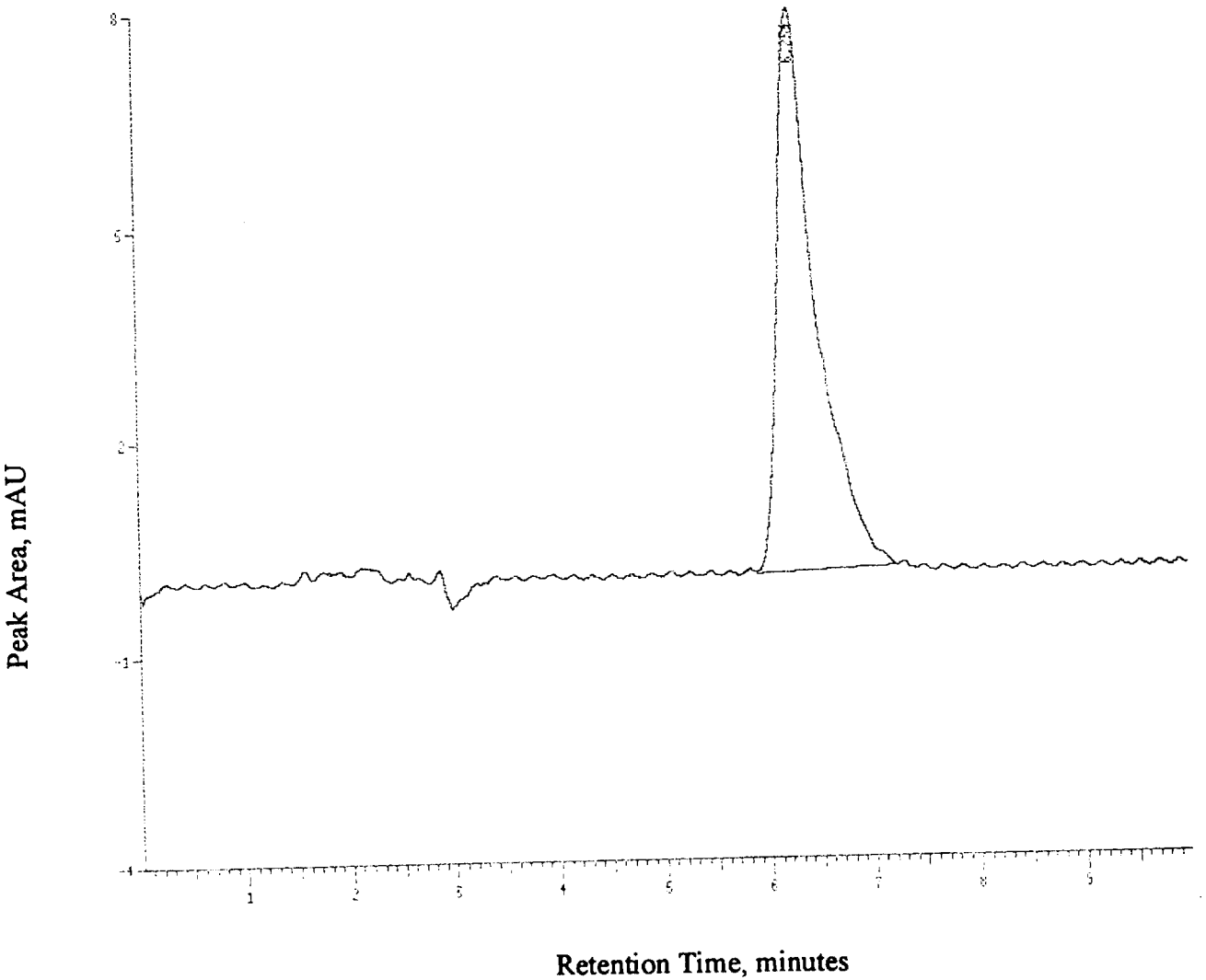

Figure 15. HPLC chromatogram of daidzein, cellulose, and iron sample.