Distribution of isoflavones and coumestrol in fermented miso and edible soybean sprouts

by

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Signatures have been redacted for privacy

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Isoflavones and coumestrol are biphenolic flavanoids which occur naturally in some legumes, especially in soybean seeds and sprouted soybeans, respectively. The isoflavones daidzein (7,4'-dihydroxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone) and the isoflavan coumestrol have been shown to have estrogenic/antiestrogenic effects in some mammals (Bickoff, 1957). Due to the wealth of findings regarding the health effects of isoflavones and coumestrol, these compounds are currently being studied by numerous researchers. Since soy foods are common dietary items and they are easy to administer in clinical trials, it is relevant for researchers and consumers to know the content of these biologically active substances in foods. As more is known about the benefits and risks associated with isoflavones and coumestrol, it becomes more important for food producers, processors, and manufacturers to know the effects of their practices on the isoflavone and coumestrol content of their final products.

Processing is known to affect the quantity and forms of isoflavones found in soymilk, tofu, and soy ingredients such as soy protein concentrate and soy isolate (Wang and Murphy, 1994a). Current research is delineating the isoflavone content of human foods which are composed primarily of soy, although very little has been published on the effect on processing on the content or distribution of isoflavones or coumestrol in soy foods. Some data has

been published on the isoflavone content of fermented soy foods such as soy sauce and miso and on the coumestrol content of soybeans and soy sprouts. Only one source (Ikeda *et al.*,1995) shows changes in isoflavones over the course of miso fermentation, and I have found no complete time course of isoflavone and coumestrol changes over the 7-day growth period from soybeans seeds to sprouted soybeans.

This study charts the changes in concentration of twelve forms of the isoflavones daidzein, genistein, and glycitein in Japanese-style miso from the beginning dry soybeans, through eight weeks of fermentation. Also examined is the increase of coumestrol content in soybean seeds sprouted for seven days.

LITERATURE REVIEW

Sources and Chemistry of Isoflavones and Coumestrol

Sources and functions of isoflavones and coumestrol

Isoflavones and coumestrol are biphenolic flavonoids which are found primarily, and almost exclusively, in legumes. The highest concentrations of isoflavones are found in soybeans. Wang and Murphy (1994b) found 1.3 to 4.2 milligrams per gram of total isoflavones in 10 varieties of soybean seeds. Coumestrol is more prevalent in clover and alfalfa, but is also present in sprouted soybean seeds. Franke *et al.* (1994), for instance, reports 281 µg/g of coumestrol in clover sprouts.

Kosslak *et al.* (1987) identified that daidzein and genistein are the major components in soybean root extract which induced *nod* gene expression in *Bradyrhizobium japonicum*, where they are involved in the synthesis of N_2-fixing root nodules in the soybean plant (*Glycine max*). Zhang and Smith (1995) explain, "An early phase in the nodulation process involves the selective attachment to and penetration of the plant root by the [*B. japonicum*] bacterium. The first step of this phase is probably the release of flavonoid signal molecules that bind the bacterial symbiont and trigger the coordinated expression of a series of bacterial nodulation genes called *nod* genes."

Daidzein is the immediate precursor of the pterocarpan phytoalexins, the glyceollins. When cotyledon tissues are infected, the daidzein and genistein conjugates are rapidly hydrolyzed at the infection front to their free forms. Glyceollin synthesis proceeds and the pathogen is contained within 48 hours (Graham *et al.*, 1990). Barz and Welle (1992) describe that constitutively produced isoflavones are preinfectional inhibitors in plants, offering protective barriers against microbes, "whereas pterocarpan phytoalexins are active plant defences directed at specific invading pathogens." Miller *et al.* (1994) demonstrated that daidzein was produced in soybean cotyledon wound droplets in response to β-glucans synthesized by *B. japonicum.*

Popravko *et al.* (1987), in a review on isoflavonoids and coumestans in Trifolieae, cite research showing that coumestans were not anti-fungal agents. However, these authors also cite the 1971 study of Wong and Latch which shows that coumestrol concentrations increased considerably when white clover plants were infected with pathogenic fungi.

Structures and chemical properties of isoflavones and coumestrol

Isoflavones are the most common of the flavonoid subgroup isoflavonoids (Wong, 1975, p. 746), which also includes coumestans. Coumestrol (6',7-dihydroxybenzofuro [3',2',3,4] coumarin) is the dihydroxylated form of the coumaranocoumarin structure, which has the highest possible oxidation level

for the isoflavonoid skeleton (Wong, 1975, p. 780). Flavonoid aglycones contain 15 carbon atoms in a C6-C3-C6 configuration; two aromatic rings linked by a 3-C unit may or may not form a third ring (Markham, 1982, p. 1). The O-glycoside forms of flavonoids are more common in plants than the aglycones. One or more of the flavonoid -OH groups is bound to a sugar or sugars by an acid-labile hemiacetal bond. Glycosylation makes the flavonoid less reactive and more water soluble, so flavonoids can be stored in the cell vacuole, where they are usually found (Markham, 1982, p. 5). Glucose is most commonly involved.

> Glycosides occasionally exhibit one further modification, that of acylation. Acylated glycosides have one or more of their sugar hydroxyls derivatized with an acid such as acetic or ferulic. The bond in this case is an ester bond, the acid effectively being esterified by the sugar (Markham, 1982, p. 6).

Isoflavones can be distinguished from flavones and isoflavanones by UV and NMR spectroscopy. Isoflavones have intense absorption at ~255-275 nm and a less intense band at ~ 310-330 nm (Wong, 1975, p. 746). Flavonoid aglycones have chemical properties of phenolics, so they are slightly acidic and will dissolve in alkali. If left in alkali, with oxygen, many will degrade. Carrying unsubitituted -OH groups or a sugar, they are polar. They are moderately soluble in ethanol, methanol, butanol, acetone, dimethyl sulfoxide, dimethyl formamide, and water. Isoflavone aglycones are more soluble in ether and chloroform (Markham, 1982, p. 15).

An aid to identification of isoflavones is that their spectra usually show bathochromic shifts of both the intense Band II absorption and the Band I shoulder or low intensity peak in the presence of NaOMe (Mabry, 1970).

> The presence of a free 5-hydroxyl group in an isoflavone can be detected by means of measuring the effect of ethanolic aluminum 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 10-15 nm. (Farmakalidis, 1984)

A clue to the presence of isoflavones in soy foods is the bitter taste (Kudou *et al.*, 1991). Their study revealed that, "All of the isoflavone components produced intensely undesirable taste effects such as bitterness, astringency, and dry mouth feeling."

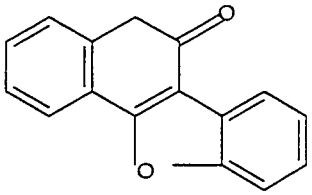
Figure 1 shows the chemical structures of coumestrol and the isoflavones and 17β-estradiol.

Physiological Effects of Isoflavones and Coumestrol

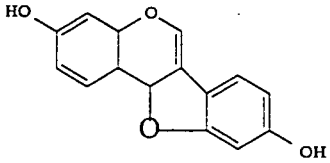
Overview of published research

Both positive and negative health effects have been attributed to soy consumption, and specifically to ingestion of isoflavones and coumestrol. Even though these compounds have only been discovered and fully characterized in recent decades, they are the subject of intense research. The *Science Citation Index* (1989-) lists 466 articles about isoflavones or coumestrol over the

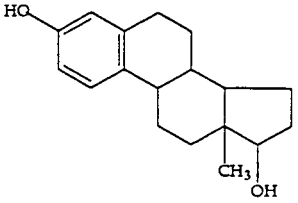
a. Coumestan



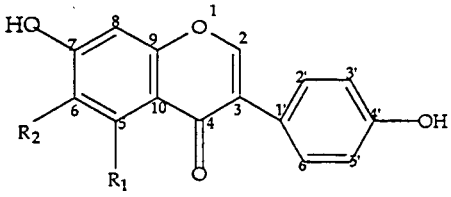
b. Coumestrol



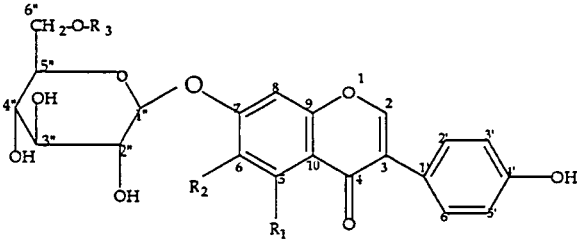
c. 17β-Estradiol



d. Isoflavone aglycone



e. Isoflavone glucoside



Compound	Abbr.	R_1	R_2	R_3	Mol. Wt.
Isoflavone aglycones:					
Daidzein	Dein	H	H		254
Genistein	Gein	OH	H		270
Glycitein	Glein	H	OCH_3		284
Isoflavone glucosides:					
Daidzin	Din	H	H	H	416
Genistin	Gin	OH	H	H	432
Glycitin	Gly	H	OCH_3	H	446
6″-O-Acetyldaidzin	AD	H	H	$COCH_3$	458
6″-O-Acetylgenistin	AG	OH	H	$COCH_3$	474
6″-O-Acetylglycitin	AGly	H	OCH_3	$COCH_3$	488
6″-O-Malonyldaidzin	MD	H	H	$COCH_2COOH$	502
6″-O-Malonylgenistin	MG	OH	H	$COCH_2COOH$	518
6″-O-Malonylglycitin	MGly	H	OCH_3	$COCH_2COOH$	532
Coumestrol	Coum				268

Figure 1. Chemical structures of coumestrol and isoflavones

years 1985 through 1995. Messina *et al.* (1994) summarize *in vitro* and *in vivo* data from 112 references which report findings regarding the link between soy intake and cancer risks. Sixty-five percent of the 26 animal studies reviewed reported protective effects, while no increases in tumor development were attributed to soy intake. Nonfermented soy foods such as soymilk and tofu were shown in epidemiological studies to be either protective or not linked to cancer risk. No consistent pattern was observed with consumption of miso or other fermented soy foods. Messina's review categorizes the physiological effects of genistein as follows: 1) possesses weak estrogenic activity; 2) specifically inhibits protein tyrosine kinases; 3) inhibits DNA topoisomerases and other critical enzymes involved in signal transduction; and, 4) in vitro, suppresses the growth of a wide range of cancer cells. The proceedings of the "First International Symposium on the Role of Soy in Preventing and Treating Chronic Disease" was published in the March 1995 supplement of *The Journal of Nutrition*. This issue discusses soy intake in relation to cholesterol reduction, heart disease prevention, and cancer prevention and treatment. Anderson *et al.*, in the August 3, 1995, issue of *The New England Journal of Medicine* focused the medical literature on the effects of soy protein on serum cholesterol concentrations in humans to 29 articles and concluded that, "the consumption of soy protein rather than animal protein

significantly decreased serum concentrations of total cholesterol, LDL cholesterol, and triglycerides."

Estrogenic effects of isoflavones and coumestrol

The estrogenic effects of isoflavones and coumestrol in animals have been recognized (Bradbury and White, 1951; Bickoff *et al.*, 1957) for over 4 decades. Messina *et al.* (1994) credit Folman and Pope (1966) with first suggesting that isoflavones may act as antiestrogens. Acting as an agonist, genistein may bind to an estrogen receptor and produce an effect resembling that of the endogenous hormone. As an antagonist, genistein will bind to the receptor without producing a biological response. Binding of coumestrol, daidzein, and genistein to estradiol receptors was examined by Verdeal *et al.* (1980). More recently, Cassidy *et al.* (1995) connected lengthening of the menstrual cycle to consumption of soy foods with known isoflavone concentrations. The study was conducted on 15 premenopausal women who lived in a metabolic suite for 4 to 6 months. Baird *et al.* (1995) did not find estrogenic effects from a soy supplemented diet in a study of 97 postmenopausal women. Lu *et al.* (1996) reinforce Cassidy's findings with results suggesting that ingestion of soymilk with known concentrations of daidzein and genistein led to a reduction of circulating ovarian steroids and adrenal androgens

and increased menstrual cycle length, which may partly explain a decreased risk of breast cancer in women who consume soy.

Effects of soy phytoestrogens on males

Males have been the subjects of phytoestrogen research studies. Lu *et al.* (1995) determined, from urinary recovery data from a one-month study of 6 males in a metabolic unit, that absorption half-lives of daidzein and genistein increased, indicating prolonged tissue exposure to these compounds and thus a possible enhancement of their cancer-protective effects. An epidemiological study by Severson *et al.* (1989) looked at the link between diet and prostate cancer in men of Japanese ancestry in Hawaii and found a slight decrease in risk of prostate cancer with increased consumption of tofu, but an increase with consumption of miso soup. Makela (1995) concluded from marginal or no effects on adult males given a soy diet that either the protective effects must follow from exposure to isoflavones in the neonatal period, or that "the chemopreventive action of soy is not due to antiestrogenicity of soy-derived phytoestrogens."

Isoflavones and cancer

The 1994 review by Messina *et al.* tabulates 40 epidemiological studies which tested for correlations between soy food intake and risk of various

cancers. A statistically significant association was found for cancers of the breast, colon, rectum, lung, stomach, esophagus, and gall bladder. While earlier hypotheses to explain the anticancer effects of isoflavones involve their antiestrogenic effects (Setchell *et al.* 1984), other mechanisms have been suggested. Messina *et al.* (1984) cites Akiyama *et al.* (1987) when he comments that, "...a marked surge of interest in soyfoods has occurred following reports showing that the soybean isoflavone genistein is a potent inhibitor of enzymes which phosphorylate tyrosine residues on key proteins involved in signal transduction events in normal and tumor cells." The activity of genistein as a tyrosine kinase inhibitor, and its lack of toxicity contrasted to synthetic analogs, creates interest in its use as a chemopreventive agent (Barnes *et al.* 1995).

Another approach was taken by Wei *et al.* (1995). They conclude, "that genistein's antioxidant properties and antiproliferative effects may be responsible for its anticarcinogenic effect." Cai and Wei (1996) observed increased antioxidant enzyme activities in mice consuming a 250-ppm genistein diet or a 50-ppm feeDing. Specifically, catalase activity was elevated in the small intestine; glutathione-S-transferase activity was increased in the skin, small intestine, liver, kidney, and lung.

Effects of coumestrol and soy diet on bone health

Given the other estrogen-related effects of isoflavones and coumestrol, these compounds might be expected to affect bone health, as well. This aspect was examined in vitro by Tsutsumi (1995), with results showing that coumestrol both inhibited bone resorption and stimulated bone mineralization. Arjmandi *et al.* (1996) found that ovariectomized rats fed with soy protein isolate (and no casein) had greater vertebral and femoral bone densities than ovariectomized rats on a soy-free casein diet. They emphasize that this study does not differentiate between the effect of the soy protein and isoflavones in the soy diet.

Inconsistent findings and risks of consumption

Although there are epidemiological associations between soy consumption and positive health effects, the findings are not definitive at this time. Likewise, the effects of fermented soyfoods are not consistent. Studies involving miso soup, for instance, are confounded by the high salt content of the miso and the use of vegetables in the soup (Messina *et al.*, 1994). The previously cited study of prostate cancer incidence among 7999 men of Japanese ancestry in Hawaii assigned a risk of 1.00 to those who consumed miso soup once per week or less. The relative risk of prostate cancer for the men who consumed miso soup five or more times per week increased to 1.24

(Severson *et al.*, 1989). Other researchers note the risks of ingestion of estrogenic isoflavones. Clarkson *et al.* (1995) report that the "adverse effects of exogenous estrogens on sexual differentiation following exposure of the fetus or the neonate" have been documented by many studies. They note that these findings constrast with the lack of "epidemiological evidence of potential developmental effects of phytoestrogens on the genital tract in populations who consume large quantities of phytoestrogen-rich soy foods." Whitten *et al.* (1995) administered coumestrol, the plant isoflavonoid with the highest estrogenic potency, to immature female rats through the milk of rat dams fed a coumestrol or a commercial soy-based diet. Ten days of treatment did not significantly alter the estrous cycle of the females later as adults, but a 21-day treatment resulted in a persistent estrous state by 132 days of age. The 10-day treatment caused significant deficits in the sexual behavior of male rats.

Fermented Japanese-style Miso

History of miso

Miso is a salty seasoning paste which is made by fermenting soybeans. The history of the first salty fermented soy food can be traced to records in China, but miso in its current form was brought to Japan from Korea, with

miso soup first occurring in the Japanese diet around the Kamakura period of 1185-1333 A. D. (Yokotsuka, 1985). Yokotsuka (1985) describes current day miso as an enzymatically degraded mixture of cooked soybeans and mold-covered rice or barley, using 8-12% salt and a small amount of water, prepared by lactic and yeast fermentations. The molds used are generally Aspergillus or Rhizopus. In 1982, the annual production of miso in Japan was estimated at 578,000 tons (Yokotsuka, 1985).

Shurtleff and Aoyagi (1983) report that a fermented soybean paste, which is a close relative of miso, was used in China in the first century B. C. Fermented soybean seasoning paste or sauce are used in other Asian countries under the names *tou-chiang* (Cantonese), *tauco* (Indonesia), *tau-cheo* (Malaysia), *tao-chio* or *tau-cheaw* (Thailand), *tuong* (VietNam), *jang* (Korea), and *miso* (Japan).

Types of miso

The three main types of Japanese miso are rice, barley, and soybean. These three come in sweet, medium, or salty versions, which can be further categorized as white, light yellow, or red. About 80% of the miso consumed in Japan is rice miso, and can be found in varieties such as White, Edo, Shinshu, or Sendai (Yokotsuka, 1985).

Traditional light-yellow miso (Shinshu miso) is a rice miso, made with soybeans : rice : salt (10 : 7.0 : 4.1) and fermented 1-3 years (Shurtleff and Aoyagi, 1983). Modern light-yellow miso accounts for over 20 percent of all miso consumed in Japan and is the most widely available miso in the United States (Shurtleff and Aoyagi, 1983). This modern version is fermented in a shorter time, eight weeks (Shimizu, 1995). The miso prepared for this study was light-yellow rice miso, with ingredient proportions of a Shinshu miso.

Traditional miso method

Traditional miso-making consists of a two-step fermentation. Rice is soaked overnight, drained, and steamed. After cooling to room temperature, it is inoculated with spores of the mold *Aspergillus oryzae* and incubated. After about 45 hours, each rice grain is covered with white mold and this product is called koji. Meanwhile, soybeans are soaked in water, cooked to soften, and cooled. Then the cooked soybeans and rice koji are mashed together with salt and a small amount of mature miso as a fermentation starter. The second fermentation takes place in 6-foot-deep cedar vats. The length of fermentation, along with the proportions of ingredients, determine the type of miso.

Small batch miso from soybeans

Shibasaki and Hesseltine (1960) mentioned having investigated the suitability of 28 different varieties of soybeans for miso. This 1960 article is only an abstract of a paper presented at a conference and does not reveal varieties tested, only saying that some varieties "absorb water and cook very unevenly," and that the color of some of the seed coats was a problem. In a follow-up study in 1961, however, they solved both problems by using full-fat soy grits from dehulled soybeans instead of the whole beans. These same researchers (1961) developed a method of making Japanese-style miso in small batches in 3000-mL capacity glass jars. They soaked soybeans for 16.5 h at 25° C. For Shinshu miso the beans were cooked for 1 h at 7 psi, cooled, and blended with the other ingredients in these proportions: (soybeans : rice : salt : water), (500 : 300 : 200 : 100). The inoculum for the second fermentation was "miso of good quality brought from Japan." After mixing the ingredients thoroughly and passing the mixture through a sausage grinder, it was packed tightly into the jars to a depth of about 8 cm. The surface was covered with parafilm, the jars were closed tightly with screw caps, and the miso was incubated at 35° C for 3 to 5 months. After fermentation, the miso was aged at room temperature for about 4 weeks.

Small batch miso from soy grits

Shibasaki and Hesseltine (1961) developed the following method in order to ensure uniform cooking of soybeans of different varieties. First they prepared soy grits from soybeans and made miso from undefatted soy grits. The soy grits were made with a roller-type crusher which produced "fine" grits, about 5-8 pieces per bean. They soaked 4,994 g of grits in 17,500 mL of tap water for 100 min., drained the excess water, and cooked the hydrated grits with steam for 1 hr at 7 psi. This process produced 10, 917 g of cooked grits with 59.3% moisture. They next mixed 1000 g of the cooked grits with 370 g koji, 200 g salt, and 70 mL tap water, and inoculated the mixture with 30 g of good miso. This mixture was packed into jars and fermented at 28° C for 7 days, followed by a 2-month fermentation at 35° C. The miso was finally aged 2 weeks at room temperature and then was ground into a paste, resulting in a yellow Shinshu miso (p. 211).

Biochemistry of miso fermentation

Shurtleff and Aoyagi (1983) explain that koji molds "produce enzymes that will later break down proteins, starches, and fats into more readily digestible amino acids, simple sugars, and fatty acids" (p. 28). Commercial rice miso starts with koji that has been prepared with a pure culture of spores of *Aspergillus oryzae* (Ahlburg) Cohn (Hesseltine and Shibasaki, 1961) or *Asper-*

gillus sojae (Yokotsuka, 1985), whereas homemade "farmhouse" miso in Japan is likely to use natural mold spores from the air. Bacteria, primarily lactic acid-formers (*Pediococcus halophilus* and *Lactobacillus delbrueckii*) transform simple sugars into organic acids, producing flavor and preventing spoilage (p. 28). Yeasts react with sugars to produce alcohols, adding to the typical miso aroma (p. 28). Alcohols and acids form esters, further enhancing aroma (p. 28-29). In the traditional method, miso ingredients are mashed together by humans treading on them with their feet in shallow wooden tubs, so of course the yeasts and bacteria inoculated in this way are not pure cultures (Shurtleff, 1983). Hesseltine and Shibasaki (1961) established that inoculation with a pure culture of *Saccharomyces rouxii* NRRL Y-2547 produced a miso with excellent flavor and pronounced good odor. Other microorganisms, such as unidentified lactic acid bacteria, could be introduced as part of the koji, since it is exposed to air during fermentation and is not heat-treated before it is blended into the other miso ingredients. Only salt-tolerant bacteria and yeasts will survive. Yokotsuka (1985, p. 234) credits Yoshii (1979) with providing the information that the a_w of *S. rouxii* is 0.78 - 0.81 and it can grow in up to 24 - 26% salt concentration. The first fermentation in the miso-making process takes place when the koji mold mycelia grow to cover each grain of rice with a white to yellowish coat. The microorganisms produce enzymes which are necessary to degrade the soybean tissues. Proteinases

degrade proteins into peptides; glutamic acid is separated from peptides by carboxypeptidase; and glutamine is separated by aminopeptidase; glutamine is then converted into glutamic acid by glutaminase (Yokotsuka, 1985).

The nutrient composition of miso is shown in Table 1.

Table 1. Composition of nutrients in 100 grams of miso

	Shurtleff and Aoyagi[a]	Ebine[b]
Type of miso	light-yellow	light-yellow, salty
Energy, kcal	155	
Moisture, %	49	45.4
Protein, %	13.5	12.5
Fat, %	4.6	6
Carbohydrate, including fiber, %	19.6	19.4
Fiber, %	1.8	2.5
Ash, %	12.8	14.2
NaCl, %	12.5	12.4
Calcium, mg	90	100
Sodium, mg	4100	
Phosphorus, mg	160	170
Iron, mg	4	4
Thiamin, mg	0.03	0.03
Riboflavin, mg	0.1	0.1
Niacin, mg	1.5	1.5
Vitamin B_{12}, µg/100 g		0.17

[a] Shurtleff and Aoyagi, 1983.

[b] Ebine, 1986.

Sprouted Soybean Seeds

Home preparation and use

Sprouted seeds have long been used in China and Korea as a winter substitute for fresh vegetables. A traditional method of sprouting soybean seeds in the residential kitchen is described in Lin (1976). Wash 120 mL of beans and soak them in 470 mL warm water for 6-8 hours. Rinse the soaked beans and put them into a plastic bag with many small drainage holes in the sides and bottom. Place four layers of moistened terrycloth on top of the bag. Rest the covered bag on a rack or a colander in a large pot and move the pot to a cool dark place (around 21° C). Rinse the beans 3 or 4 times a day, no less than every 8 hours, by filling the bag with clean water and letting it drain out. Re-moisten the terrycloth at the same time. "In about 6 to 7 days the sprouts will be [5 to 8 centimeters] long and ready to eat." (Lin, p. 92-93)

Lin (1977) notes that bean sprouts should be rinsed in a sinkful of cold water before using. During this rinsing procedure, most of the seed coats will float to the top and can be discarded (p. 92). This author includes a recipe for soybean sprout soup in which she instructs the cook to pick off and discard the root of each soybean sprout.

Commercial preparation

The commercial preparation of soybean sprouts is done by a similar method on a much larger scale. Beach (1995) recommends that a scaled-down model of a commercial system can be prepared by making a 1-inch diameter hole in the bottom edge of a 5-gallon bucket and blocking it with a stone. Then put 1½ inches of soybean seeds, not pre-soaked, in the bottom of the bucket and set it at a slight angle so it will drain. Cover the bucket loosely with plastic sheeting, place it in the dark at 68-70° F and water thoroughly 5-6 times a day with 70-74° F water. Commercially grown sprouts are likely to be shorter and thicker than home-grown sprouts due to the modification of the growth atmosphere by the addition of ethylene. Ethylene produces a thicker diameter hypocotyl (Ahmad, 1985).

Vendors who provide soybeans for commercial sprouts recommended small to medium-sized soybeans with clear or darker hilums. York and Jack are two varieties considered acceptable by vendors, who are not willing to divulge the variety of the beans they sell, for competitive reasons. One seed supplier (Gates, 1995) insisted that soybean variety is not the key to good sprouts, and that freshness of the seeds is more important, with germination decreasing substantially by the summer following autumn harvest.

Extraction and Analysis of Isoflavones and Coumestrol

Extraction and analysis methods

Published methods use UV absorbance and fluorescence for isoflavone and coumestrol detection. Wolfbeis and Schaffner (1980) reported their determination of the absorption and fluorescence characteristics of coumestrol in different pH and solvent systems and concluded that, "Fluorimetry offers an analytical method superior on all practical counts." The choice of extraction method depends on the polarity of the compounds which the researcher is extracting. Bickoff *et al.* (1958) first extracted coumestrol from Ladino clover using a solvent system which included Skellysolve C, ether, chloroform, and counter-current distribution. In 1961, Livingston *et al.* (1961) published a quantitative method using paper chromatography to measure coumestrol in fresh and dry alfalfa. Livingston's method was improved by alcohol extraction of chlorophyll prior to the paper chromatography (Knuckles *et al.*, 1975). Lookhart (1979a) evaluated the Knuckles (1975) and Livingston (1961) methods on soybeans and achieved very low recoveries, which he attributed to the lipid content of the samples. Lookhart *et al.* (1979b) used reversed-phase high-performance liquid chromatography (HPLC) to quantitate coumestrol extracted from soy sprouts, applying the 3-hour extraction method from their

1978 article. For that study, whole soybeans or fractions were first ground and hydrated with water, then ground and hydrated with methanol, and centrifuged. The supernatant was defatted with pentane, extracted with ethyl ether, concentrated by evaporation, and made up to volume with the HPLC mobile phase of (65 : 35) (methanol : water). Coumestrol peaks were integrated from a UV trace, with a fluorometer used to help identify the compound. Recovery of coumestrol from a spike added to dry sample before extraction was 64.0%.

Murphy's 1981 study introduced deactivation solvents into the extraction process, comparing 12 solvent systems made up of methanol, chloroform-methanol, acetonitrile, or acetone, each without deactivation, deactivated with 5 mL water, or deactivated with 5 mL 0.1 N hydrochloric acid. The results of that study reported 106 ± 20 ng coumestrol with acetonitrile and HCl, which had a 55% recovery. The only higher coumestrol result was 133 ± 84 ng with acetone and water, which produced more than 2 times the amount of co-extractive residue of the acetonitrile-HCl system. Murphy cites Lookhart (1980) and states, "Water considerably reduced the efficiency of the methanol extraction of coumestrol." Pettersson and Kiessling (1984) extracted coumestrol from soybean meal by first extracting the sample with petroleum ether. They re-extracted the ether with 80% ethanol, hydrated the defatted sample with water, added 3M hydrochloric acid and the ethanol re-extract, heated to

boiling, cooled, filtered, and cleaned up the filtrate with a Sep-Pak C_{18} cartridge. HPLC analysis by UV and fluorescence showed that extraction of silage with acetonitrile and HCl yielded smaller quantities of isoflavones than extraction with 75% ethanol, but this comparison was not reported for coumestrol or soybean meal. Franke *et al.* (1994) quantitated coumestrol from clover sprouts and alfalfa sprouts with HPLC and fluorescence detection after refluxing in 10 M HCl and 96% ethanol.

Nearly all of the published methods for extracting isoflavones from soy use heat, which converts malonyl glucoside forms to glucosides and acetyl glucosides. Possibly because of the unavailability of standards for the acetyl and malonyl forms of the isoflavones, most researchers do not quantitate these forms. Published data of isoflavone concentrations in foods tends to be glucosides and free forms only. It is common to see total isoflavones measured following acid hydrolysis. Ohta *et al.* (1979) identified acetyl daidzin after extracting defatted soy flour with hot ethanol for 3 hours and acetone for 2 hours. They eluted isoflavones from silica gel and Sephadex LH-20 columns with 50% water-saturated ethyl acetate and achieved HPLC separation of acetyl daidzin. Murphy's 1981 comparative study of extraction solvent systems states objectives of maximizing extraction efficiency and increasing speed of separation of isoflavones. The single extraction step requires 2 hours on a shaker, and separates daidzin, genistin, daidzein, and genistein

by HPLC in 15 minutes with a non-linear methanol-water gradient. Recoveries are reported for acetonitrile/water, acetonitrile/HCl, and acetone/HCl. Eldridge (1982a) extracted dehulled, defatted soybean flour with 80% methanol by refluxing due to its reproducibility and extraction advantages, and also investigated these solvents: 50%, 80%, and absolute ethanol; 50%, 80%, and absolute methanol; ethyl acetate, and acetonitrile. Eldridge and Kwolek (1983) compared the content of isoflavone aglycones and glucosides in full-fat and in defatted soybean meal and concluded that defatting full-fat soybean flakes does not remove isoflavones. Extraction was done by boiling samples in 80% methanol 4 hours on a steam bath. Isoflavones were measured by HPLC with a 25 to 50% linear methanol gradient in 20 minutes with *n*-butyrophenone as internal standard. Pettersson and Kiessling's method (1984) uses acid and heat and they quantitate only aglycones. Seo and Morr (1984) improved the resolution of phenolic compounds from soy by HPLC. They refluxed 1-g defatted soy flake samples in 80% ethanol, followed by a 6-hour hydrolysis at room temperature with 2N NaOH to free phenolic acids from their esters. The extracts were cleaned up on C_{18} Sep-Paks and analyzed at 280 nm with mobile phase of (A) 5% acetic acid in water, and (B) 5% acetic acid in methanol. A linear gradient of 0 to 100% B was run in 55 minutes. Farmakalidis and Murphy (1985) achieved separation of 4 forms of isoflavones (Din, Gin, AD, and AG - see abbreviations key in Figure 1) by extracting

with 80% methanol (Eldridge, 1982a) and 6 forms of isoflavones (Din, Gin, AD, AG, Dein, and Gein) and coumestrol when extracting with acetone and 0.1 N HCl (Murphy, 1981). Jones *et al.* (1989) analyzed foods from the normal British diet by HPLC, using borax-phosphate-buffered acetonitrile and water mobile phase with ethyl benzoate as internal standard. Graham *et al.* (1990) isolated 6"-O-malonyl-7-O-glucosyl daidzein and 6"-O-malonyl-7-O-glucosyl genistein from soybean cotyledons after extraction with 80% ethanol "immediately upon thawing after storage at -80° C" and analyzing by HPLC with acetonitrile and water mobile phase. Kudou *et al.* (1991a) report the isolation and identification of 6"-O-acetyl glycitin and (1991b) 3 malonyl isoflavone glycosides (6"-O-malonyldaidzin, 6"-O-malonylgenistin, and 6"-O-malonylglycitin). Soybean seed hypocotyls were extracted with 70% ethanol at room temperature, extracted with 1-butanol and water, fractionated over a Sephadex LH-20 column, and separated on HPLC. MGly was separated from 11 other forms of isoflavones in 50 minutes on a YMC-pack ODS-AM-303 column (250 x 4.6 mm) with a linear gradient from 15 to 35% acetonitrile with 0.1% acetic acid. UV absorption was measured at 260 nm. The 1993 analyses of soy foods by Coward *et al.* are based on extraction of isoflavones into 80% aqueous methanol for 1 hour at 60° C. HPLC mobile phase was 0 to 46.4% acetonitrile with 0.1% trifluoroacetic acid. Fluorescein was added as internal standard, and only Gin, Din, Gein, and Dein are reported. Franke *et*

al.(1994) extracted legume foods and ingredients by refluxing a 1-g sample in 10 mL HCl : 96% ethanol (1 : 4) with flavone as internal standard. This study reported only Dein, Gein, Coum, formononetin, and biochanin A, and recoveries of these compounds with the proposed method ranged from 94 to 105%. Wang and Murphy (1994a) determined the quantity of Dein, Gein, and Glein and their glycosides and acetyl and malonyl conjugates in soybean paste and miso without defatting by using the extraction method of Murphy (1981) with (sample : acetonitrile : water : 0.1 N HCl) ratios of (1 : 5 : 0 : 1). After stirring for 2 hours at room temperature, the mixture was filtered, the filtrate was evaporated, and the dried extract was redissolved in 80% aqueous methanol. A study published in Japanese (Ikeda *et al.* 1995) measured Gin, Din, Gein, and Dein in fermenting miso, and in tempeh and defatted soybeans. They defatted miso and extracted three times with 60-70° C methanol before analyzing by HPLC. A more thorough composition study of soybean seeds was reported by Padgette *et al.* (1996). They extracted free isoflavones with ethanol and dilute HCl, determined total isoflavones by refluxing the extract with 4 M HCl, and reported bound isoflavones as the difference.

Isoflavone Content of Miso

Table 2 displays the isoflavone content of various types of miso and soybean paste, as reported by three research groups. In large part due to the

choice of extraction methods, as reported in the previous section, only Wang and Murphy (1994) were able to measure all 12 forms of Dein, Gein, and Glein. It is important to note that the isoflavone totals reported by Coward *et al.* are arithmetic sums of the aglycone and glucoside values. Wang and Murphy normalized the values for the 9 glucosides before summing, to remove the effect of the molecular weight of the glucose moities and their substituent groups. Ikeda *et al.* (1995) measured only Dein, Gein, Din, and Gin, and reported the quantity of both forms normalized as aglycone equivalents. Their method was not sensitive enough to detect glycitein.

Isoflavones in Tempeh

Tempeh is an Indonesian food product consisting of cooked soybeans which are inoculated with a Rhizopus mold and incubated for 1 - 3 days. As a fermented soy food, it provides another source of information about isoflavones changes resulting from microbial activity. Wang (1995) reported that fermentation of tempeh with *Rhizopus oligosporus* did not generate major differences in total isoflavone content from cooked soybeans. The fermentation increased the proportion of aglycones to glucosides. Wuryani (1995) compared the isoflavone content of tempeh prepared with a strain of *R. oligosporus* and a strain of *R. oryzae.* Gein and Dein concentrations increased in

Table 2. Isoflavone content of miso and soybean paste (μg/g)

Product	Aglycone			Glucoside			Acetyl			Malonyl			Total			
	Dein	Gein	Glein	Din	Gin	Gly	Din	Gin	Gly	Din	Gin	Gly	Dein	Gein	Glein	Total
bean paste[a,b]	271	183	54	nd	96	21	1	2	nd	nd	nd	19	272	245	77	593
honzukuri miso[a,b]	34	93	15	72	123	18	1	11	nd	nd	nd	22	143	224	23	390
miso[c,b]	345	497		35	43											920
rice miso[c,b]	71	136		0	198											404
barley miso[c,b]	185	239		142	155											721
soybean paste[c,b]	197	251		44	78											570
soybean paste, rice[c,b]	103	108		85	66											362
soybean paste, wheat[c,b]	105	124		94	110											433
miso[b,d]	516	745		54	64											1379
rice miso[b,d]	127	242		0	353											721
barley miso[b,d]	306	396		235	258											1195
Shiromiso soup mix[c,b]	108	170		163	267											708
Akamiso soup mix[c,b]	136	173		254	319											882
soybean paste[c,d]	404	514		90	160											1168
soybean paste, rice[c,d]	166	174		136	106											582
soybean paste, wheat[c,d]	210	248		189	220											867
mamemiso[e,f]	550	850		0	17											

[a]Wang and Murphy, 1994.

[b]As-is sample.

[c]Coward, *et al*.1993.

[d]Dry sample.

[e]Ikeda, Ohta, and Watanabe, 1995.

[f]After 12 days of fermentation.

both cases while Gin and Din decreased, due to hydrolysis of the glycosides. The R. oligosporus converted more glucosides to aglycones in the 36-hour incubation period.

Isoflavones and Coumestrol in Sprouted Soybean Seeds

Table 3 is a survey of published isoflavone and coumestrol values for germinated soybeans. Note that only one researcher (Murphy, 1982) reports both coumestrol and isoflavone data for soybean sprouts, measuring only four forms of the isoflavones. While there is variation in coumestrol content

Table 3. Isoflavone and coumestrol content of germinated soybeans (μg/g)

Variety	Fraction	Days	Dein	Gein	Din	Gin	Coum
Unknown	Sprouts[a,b]						71.1
Amsoy	Whole beans[c,b]	0					0.02
Amsoy	Whole beans[c,b]	3					3.94
Columbus	Whole beans[c,b]	0					0.09
Columbus	Whole beans[c,b]	7					0.75
Amsoy	Hulls[c,b]	3					15.21
Amsoy	Whole beans[d,e]	0	1	40	117	747	0
Amsoy	Soy sprouts[d,e]	5	19	78	92	403	7
Unknown	Hulls[f,b]	0	nd	74.1			nd

[a]Knuckles, deFremery, and Kohler, 1976

[b]Freeze dried before analysis

[c]Lookhart, Finney, and Finney, 1979

[d]Murphy, 1982

[e]Dried in a convection oven before analysis

[f]Franke, *et al.*, 1994

due to soybean variety, both Murphy and Lookhart *et al.* measured an increase in coumestrol after germination.

Statement of Research Plan

This study was done to add to the base of knowledge about the effect of processing of soy food products on the content and distribution of coumestrol and the isoflavones daidzein, daidzin, genistein, genistin, glycitein, glycitin, and the acetyl and malonyl conjugates of the glucoside forms. Specifically, this research will chart the changes in isoflavone content and distribution in light-yellow rice miso from the raw ingredients through 84 days of fermentation. I will also examine the changes in coumestrol content of soybeans from 0 through 7 days of germination. In addition to looking at changes in the forms of the isoflavones, mass balance studies will determine whether the miso-making process or sprouting of soybeans changes the total concentration of normalized isoflavones.

MATERIALS AND METHODS

Miso Preparation and Sampling

Miso preparation was based on the methods of Shibasaki and Hesseltine (1961) and Shimizu (1995) and modified to laboratory scale.

Soybeans used for miso

Vinton 81 soybeans, purchased from the Iowa State University (ISU) Committee for Agricultural Development, were grown locally in 1994. The soybeans were stored in the original 20-kilogram bag, enclosed in a plastic container, at -29° C. About 6 kilograms of beans were removed from the bag and allowed to equilibrate to room temperature before weighing and making them into grits.

Soy grits preparation

The soy grits were prepared in the ISU CCUR (Center for Crop Utilization Research) Dry Pilot Plant. The seeds were passed once through a cracking roll (Ferrell-Ross, Oklahoma City, OK), producing approximately 6 - 8 pieces per bean. Those pieces were then fed once through the KICE Model 6F6 cascade aspirator (Kice Industries, Inc., Wichita, KS) for dehulling at Magnehel-

lic setting 1000. The aspirator removed nearly 100% of the hull material, plus a large amount of fines, together making up more than 18% of the weight of the soybeans. The soy grits, containing 10.72% moisture, were double bagged in plastic and stored at -29° C until used in miso.

Miso preparation

Soybean grits, described in the previous section, were soaked in cold tap water (500 g grits : 1000 mL water) for 4 hours at room temperature, in a one-gallon glass jar, closed with a screw cap. The jar of soaked grits was autoclaved (AMSCO, American Sterilizer, Erie, PA) at 121° C. After 60 minutes the autoclave was exhausted slowly and the cooked grits were allowed to cool to room temperature.

The fermentation mixture was blended in the gallon jar containing the cooked grits by stirring in with a long-handled spoon the following in the order given: (1) 50 g of starter miso (Cold Mountain Light Yellow miso, Miyako Oriental Foods Inc., Baldwin Park, CA, purchased locally), dispersed in 150 mL of sterile tap water; (2) 240 g Morton Table Salt (Morton Salt, Chicago, IL, contains NaCl plus sodium silicoaluminate, an anticaking agent), and; (3) 500 g rice koji (Cold Mountain firm granular rice koji, Miyako Oriental Foods Inc., purchased locally). The mixture was stirred thoroughly. The upper inside walls of the jar were carefully wiped free of the fermenta-

tion mixture with Kim-Wipes (Kimberley-Clark Corporation, Roswell, GA) moistened with sterile tap water. The surface of the mixture was covered with one layer of Parafilm (American National Can, Neenah, WI), the jar was closed tightly with its screw cap, and it was placed in an incubator in the dark at 31° C for 84 days.

Controls

Two one-quart jars (Mason canning jars, sealed with Ball caps and rings) of grits (125 g soy grits : 250 ml tap water) were prepared, autoclaved, and incubated along with the miso and incubated, sealed, as controls. One jar was sampled after 24 hours; the second was sampled after 5 days of incubation.

Miso sampling

Cooked grits were sampled just prior to mixing in the other ingredients. Miso was sampled immediately following preparation (day 0), then at days 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 22, 29, 36, 43, 50, 57, 64, and 84. For sampling, the jar was removed from the incubator, the screw cap and Parafilm were removed, and the surface was flooded with nitrogen gas. A sterile long-handled spoon was used to mix the miso thoroughly, under a gentle stream of nitrogen gas. After removing a sample of about 20 g, the Parafilm was

replaced. If the inside of the jar became smeared with miso during sample removal, it was wiped clean with Kim-Wipes moistened with sterile water or ethanol. The samples were stored in plastic cups with screw caps at -29° C until extracted.

Commercial miso samples

Samples were taken by a commercial miso maker from the initial fermentation mixture, and at 7-day intervals from the fermenting miso, for a total of 57 days. The samples were shipped at ambient temperature, from their source to this lab, by air express during hot summer weather. Upon receipt here, the samples were stored at -29° C until prepared for extraction. Samples of their starting materials were also provided, including organic soybeans of an unknown cultivar, rice koji, and sea salt.

Soybean Sprouts Preparation

The procedures used to grow soybean sprouts were modeled after the method used by a commercial sprouts grower (Beach, 1995), adapted for laboratory scale production.

Soybeans used for sprouting

The soybeans selected for sprouting were donated by Dr. Charles Hurburgh, ISU, from the Iowa Gold Study. They were Dekalb 291, 1995 crop year, grown in Arcadia, Iowa, test plots. A 15% moisture level was determined in Dr. Hurburgh's lab by near infrared spectroscopy (Infratec 1225, Tecator A/B, Hoogenas, Sweden). The Dekalb seeds were chosen for the following reasons: 1) the variety and growth conditions are known; 2) the size is similar to seeds sprouted by commercial growers; 3) the seeds have light hilums; and, 4) cultivars recommended by seed suppliers had very low germination rates due to an early 1995 autumn freeze.

Table 4 shows the results of a preliminary experiment of the germination rate and sprout length of several other cultivars. Eight lots of 20 soybeans each were sprouted on moistened paper towels for five days.

Table 4. Length of soybean sprouts five days after germination

	Length (cm)					
Cultivar	0[a]	< 3	3-5	>5-8	>8-10	>10
Pioneer 9091	1	5	2	4	8	0
Vinton	1	10	4	2	3	0
Chesapeake	2	10	2	3	3	0
Ohio	0	11	3	2	4	0
Stine 0653	0	13	2	1	4	0
Stine 2170-X	1	12	2	4	1	0
Unknown[b]	3	9	2	5	0	0
Unknown[c]	0	6	2	3	5	4

[a] Did not germinate.

[b] Organically grown food grade soybeans purchased locally.

[c] Obtained from a commercial sprout grower (Beach, 1995).

Sprouting of soybeans

Intact dry soybean seeds, 120.0 ± 0.1 g, were placed in each of 21 "paper cans" (1-quart capacity cylinders, 8.8 cm outside diameter, 16.8 cm high, Fonda, Union, N J). The seeds were neither pre-soaked nor surface sterilized. A single drain opening of approximately 3 mm diameter was punctured into the side of each can, about 1 cm up from the bottom edge of the can. A paper container (1-cup volume dish, 6 cm high, 7.5 cm bottom diameter, 9.2 cm outside top diameter, Sweetheart, Heartland Paper, Cedar Falls, IA) with 64 drainage holes punctured in a spiral pattern with a 4-tined dinner fork was suspended inside the top of the paper can to serve as a water dispenser and as a cover to limit evaporation.

The 21 cans (3 rows of 7 cans/row) were placed on a wire shelf in the dark inside a growth chamber (Sherer, Boone, IA) in the ISU Horticulture Dept. The temperature was controlled at 19.5 - 20.0° C. Tap water was equilibrated to 19.5 - 20.0° C inside the growth chamber, and 500 mL was gradually added to the water dispenser cup on each can of sprouts at four-hour intervals until harvest.

Harvest and sampling of soybean sprouts

The cans of soybeans were labeled randomly with numbers 1-21 before being placed in the growth chamber. A random sequence of the numbers

was prepared by a blind drawing. This sequence was used as the harvest order for the cans of beans/sprouts.

Cans of beans drawn for harvest were not given the sixth watering of the 24-hour period ending in their harvest. The contents of a harvested can were weighed, sealed in two layers of zip-closed plastic one-gallon capacity storage bags, and frozen at -29° C until freeze dried. Following harvest of all 21 cans, the complete contents of all 21 cans were freeze-dried in the plastic storage bags.

Moisture content of the sprouted beans was calculated as the difference between the harvest weight and the weight after freeze-drying.

Isoflavone and Coumestrol Analysis

Reagents

Unless otherwise noted, all reagents were HPLC grade from Fisher Scientific Company (Pittsburgh, PA), and water was filtered through a Milli-Q Reagent Water System (Millipore Co., Bedford, MA).

Standards

Standards were purchased from commercial sources or purified from soy material or synthesized in this laboratory. Table 5 shows the sources and

Table 5. Sources of standards used in HPLC analyses

Standard	How used	Source
coumestrol	std. curve	ACROS Organics, div. of Fisher Scientific, Pittsburgh PA
genistein	std. curve	synthesized in this lab by T. Song
daidzein	std. curve	Sigma
genistin	std. curve	purified in this lab by H. Wang
daidzin	std. curve	purified in this lab by H. Wang
glycitin	std. curve	purified in this lab by H. Wang
acetyl genistin	peak i.d.	purified in this lab by H. Wang
acetyl daidzin	peak i.d.	purified in this lab by H. Wang
malonyl genistin	peak i.d.	purified in this lab by H. Wang
malonyl daidzin	peak i.d.	purified in this lab by H. Wang
acetyl glycitin	(not used)	no standard available; calculated std. curve based on Gly
malonyl glycitin	(not used)	no standard available; calculated std. curve based on Gly
2,4,4'-trihydroxy-deoxybenzoin	reference std.	synthesized in this lab by T. Song

uses of standards for each of the 14 compounds analyzed as part of this study. Table 6 contains standard curve information.

Sample preparation and extraction

Samples of miso and cooked soybean controls were ground to a smooth paste with a mortar and pestle. Mature soybeans and harvested sprouted soybeans were first freeze-dried and then ground to a powder in a coffee grinder (Mr. Coffee, Inc., Bedford Heights, OH). A 4.0000 g ± 0.1000 g as-is miso sample or a 2.0000 g ± 0.0100 g of freeze-dried soybean or soybean sprouts powder was weighed into a 125-mL Erlenmeyer flask. Solvents used for miso extraction, in order of addition, were: 5 mL water (optimum amount

Table 6. Standard curves

Standard	Purity (%)[a]	(μg/mL) = slope x (peak area) + (y-intercept)	Coefficient of determination	Concentration range (μg/mL)
Coumestrol	93.83	$Y = 3.494 \times 10^{-6} X + 0.1364$	0.998	1 - 21
Daidzein	98.85	$Y = 0.64 X + 1.26$	0.999	10 - 200
Daidzin	91.75	$Y = 1.19 X + 0.74$	0.999	10 - 150
Genistein	100.00	$Y = 0.59 X + 0.95$	0.999	10 - 200
Genistin	98.37	$Y = 0.92 X + 1.91$	0.999	5 - 200
Glycitin	92.29	$Y = 0.89 X + 1.20$	0.999	5 - 60
Reference Std.	96.72	$Y = 4.85 X - 1.55$	0.999	20 - 200

Purity was measured as per cent of area integrated.

determined by experiment), 20 mL acetonitrile, and 2 mL of 0.1 N hydrochloric acid (Murphy, 1981). Extraction solvents added to freeze-dried soybean or soybean sprouts powder were: 5 mL water, 10 mL acetonitrile, and 2 mL of 0.1 N HCl. The flask was stoppered and extracted for 2 hours at 300 rpm on a platform shaker (innova 2000, New Brunswick Scientific Co., Edison, NJ) at room temperature. Miso sample slurry was vacuum filtered through a Whatman 40 filter paper (Whatman 42 for soybean and sprout samples). The filtrate was quantitatively transferred to a 250-mL round-bottomed flask and rotary evaporated to ≤ 1 mL volume at 30-34° C (Büchi, Brinkmann Instruments, Inc., Westbury, NY). The isoflavone-containing material was then redissolved in (80 : 20) (methanol : water), and transferred quantitatively to a 10-mL volumetric flask containing 100-200 μL of reference standard solution. After mixing well, 1 mL was taken up in a 1 cc Tuberculin

syringe (Becton Dickinson & Company, Franklin Lakes, NJ) and expelled through a 0.45 μm PTFE filter unit (polytetrafluoroethylene, Alltech Associates, Inc., Deerfield, IL) into an autosampler vial.

Coumestrol was extracted from samples of freeze-dried sprouted soybeans using the method described above, with a solvent system of (sample : methanol : 0.1N HCl) of (2 : 10 : 2) and filtered through Whatman 42 paper. The extracted solids, after rotary evaporation, were redissolved in 100% methanol.

High-performance liquid chromatography (HPLC)

Isoflavones The HPLC method was developed based on Wang (1994a), modified to shorten cycling time. All isoflavone analyses were done on a Beckman HPLC system (San Ramon, CA) consisting of: 126 dual pump solvent module; 507 autosampler with column heater set at 32° C and 20μL loop; 168 UV detector with photodiode array; Beckman System Gold software, Version 8.10; and IBM Personal Computer 350-466 DX2. This was a new system which had not been used for isoflavone analyses. This researcher set up the methods, formats, and analysis parameters on the Beckman software. The column used was a YMC-pack ODS-AM-323, 10 μm, 25 cm x 10 mm i.d. (YMC, Inc., Wilmington, NC) The mobile phase was (A) water with 0.1% glacial acetic acid, and (B) acetonitrile, with 0.1% glacial

acetic acid. A gradient was run as shown in Table 7. Chromatographic peaks were integrated at 254 nm, with a peak threshold of 0.00500, peak width of 0.3000, and minimum peak height of 0.00200. Scans were collected over 200-350 nm, at a data rate of 2 hz.

Table 7. HPLC mobile phase gradient for isoflavone analysis

Time	% B	Duration	mL/min	Duration
0.00	15		1.0	
4.75	15		1.5	0.50
5.00	29	30.90	1.5	
36.00	35	8.00	1.5	
45.00	15	1.00	1.0	0.50
60.00			end	

Isoflavone recovery experiment One sample was taken from each of three replicate batches of miso at 57 days of fermentation. Approximately 2 grams of each, to the nearest 0.1 mg, was spiked with 200 µL of 824 µg/mL daidzein standard, 200 µL of 1766 µg/mL of genistein standard, and 200 µL of reference standard. Recoveries of the spikes were, respectively, 97%, 101%, and 100%.

Coumestrol The HPLC system and method used for isoflavone analysis was not suitable for analysis of coumestrol at the small concentrations present in the soy sprouts. Since coumestrol is more readily detected by fluorescence, the HPLC system used for sprouts analysis consisted of: 2 Beckman 110B pumps; Beckman 502 autosampler; Waters Model 470 fluorometer

(Waters Corporation, Milford, MA), gain = 10X. Wavelengths were set at exλ=365 nm, emλ=420 nm (Petterson and Kiessling, 1984); NEC PC-8300 controller (NEC Home Electronics U. S. A. Inc., Wood Dale, IL) with Beckman software; Beckman 427 integrator, atten = 1024. The analytical column is a YMC Pack, A-303 S-5 120A ODS, 250 x 4.6 mm i.d.

The flow rate was 1.0 mL/min. with mobile phase of 50% methanol in water plus 0.1% acetic acid, with a 15.0 minute linear gradient to 100% methanol at 0.10 minutes, and a 1.0 minute linear return to 50% methanol at 18.0 minutes. Coumestrol eluted at 12.6 minutes.

Statistical Analysis of Data

Statistics were calculated using Microsoft Excel, Version 5.0 (Microsoft Corporation). Means, standard deviations, and regression analysis were used. ANOVA was used to calculate p -values, with < 0.05 chosen as the level of statistical significance. On Tables 16 and 21 differences in means were determined by ANOVA and GLM using the SAS package (SAS Institute, Inc., Box 8000, Cary, NC), also at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Observations

Miso fermentation

Three batches of miso were prepared and fermented as described in the Materials and Methods section. For comparative purposes, the initial ingredients and samples of miso at weekly intervals during fermentation were provided for analysis by a commercial miso maker. Qualitative characteristics (i.e., color, odor, and texture) of the miso prepared in this lab were very similar to the commercial miso. The initial fermentation mixture was a thick paste with a typical tan color and cooked soybean odor. At the end of one to two weeks of fermentation, an ethanol odor was apparent when the jar lids were removed for sample removal, and there was a recognizable characteristic miso odor. By about day 15, the fermenting paste had become golden-colored and small amounts of brown liquid were collecting in air pockets at the bottom of the jars and on top of the mixture. The ethanol odor became stronger, with fruity notes, and on day 22 of one of the three batches and on day 8 of another, the Parafilm was elevated about 1 cm above the surface of the miso due to gas entrapment. By 35 days of fermentation the color of the miso had become a darker golden brown. At day 57, the 8-week harvest

point for light-yellow Shinshu miso, the aroma of all three batches of miso was the full rich typical aroma of light-yellow miso. The fermentation was continued until day 84, at which time the miso had become deep brown and the amount of dark brown soy sauce-like liquid collected in the interstices had increased but was still less than about 1% of the total volume of the contents of the containers.

Sprouting soybean seeds

The soybeans were not soaked prior to incubation in the growth chamber, which could be expected to slow their growth, at least initially. The size and level of development of the sprouts after 7 days of growth, however, indicated that their growth rate was not slower than any of those shown in Table 4. Most of the full-sized sprouts harvested on day 7 had the beginnings of side roots, which is normal for 7-day soybean sprouts. Due to the method used to grow them, it was possible to measure the length of the sprouts. Typical lengths are reported in Table 8. The overall appearance of the sprouts was very similar to that of commercial soy sprouts. According to Beach (1995), commercial soy sprouts are grown for 6-7 days before shipping to consumers.

Some mold growth was observed in the bottom of the containers of the last 3 cans of sprouts harvested.

Table 8. Typical lengths of soybean sprouts

	Age of Sprouts at Harvest (hours)						
	24	48	72	96	110	134	168
Length	no sprout growth	0 to 1.5 cm past the bean	3 to 4 cm bean + sprout	5 to 6 cm bean + sprout	8 to 10 cm bean + sprout	10 to 16 cm bean + sprout	16 to 20 cm bean + sprout

Isoflavone Content and Distribution

Isoflavones and coumestrol were identified by HPLC elution time compared to standards, as well as by comparison of the ultraviolet scans to standards. UV chromatograms of miso at 1 and 57 days of fermentation follow as Figures 2 and 3. Figure 4 is a fluorescence chromatogram of the coumestrol standard. Figures A-1 through A-7 in the Appendix are UV scans of isoflavones and coumestrol standards from the Beckman HPLC system. The 12 isoflavones measured in the miso and sprouts studies accounted for 96% of the UV-absorbing material detected in miso at day 1; 94% in miso at day 57; 85% in Dekalb 291 soybean seeds; and 86% in sprouted soybeans.

Tables 9, 10, and 11 and Figures 5, 6, and 7 show the content and distribution of isoflavones in the miso over the course of 84 days of fermentation. Table 12 shows the normalized concentrations of daidzein, genistein, and glycitein, which are the concentrations of these three compounds that the

Figure 2. Chromatogram of miso fermented 1 day

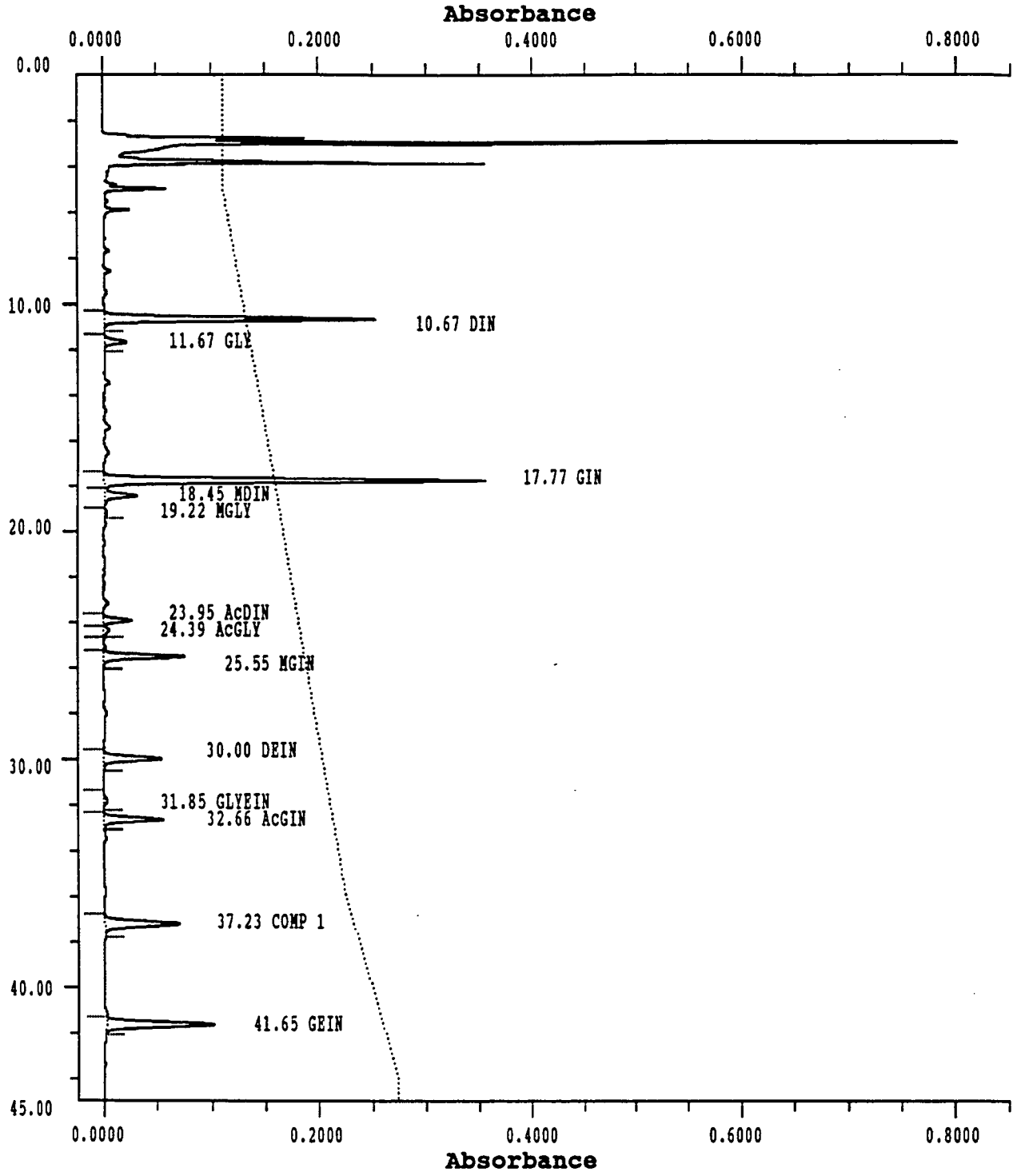

Absorbance
0.0000
0.2000
0.4000
0.6000
0.8000
0.00
10.00
20.00
30.00
40.00
45.00
10.67 DIN
11.67 GLY
17.77 GIN
18.45 MDIN
19.22 MGLY
23.95 AcDIN
24.39 AcGLY
25.55 MGIN
30.00 DEIN
31.85 GLYEIN
32.66 AcGIN
37.23 COMP 1
41.65 GEIN
Absorbance

Figure 3. Chromatogram of miso fermented 57 days

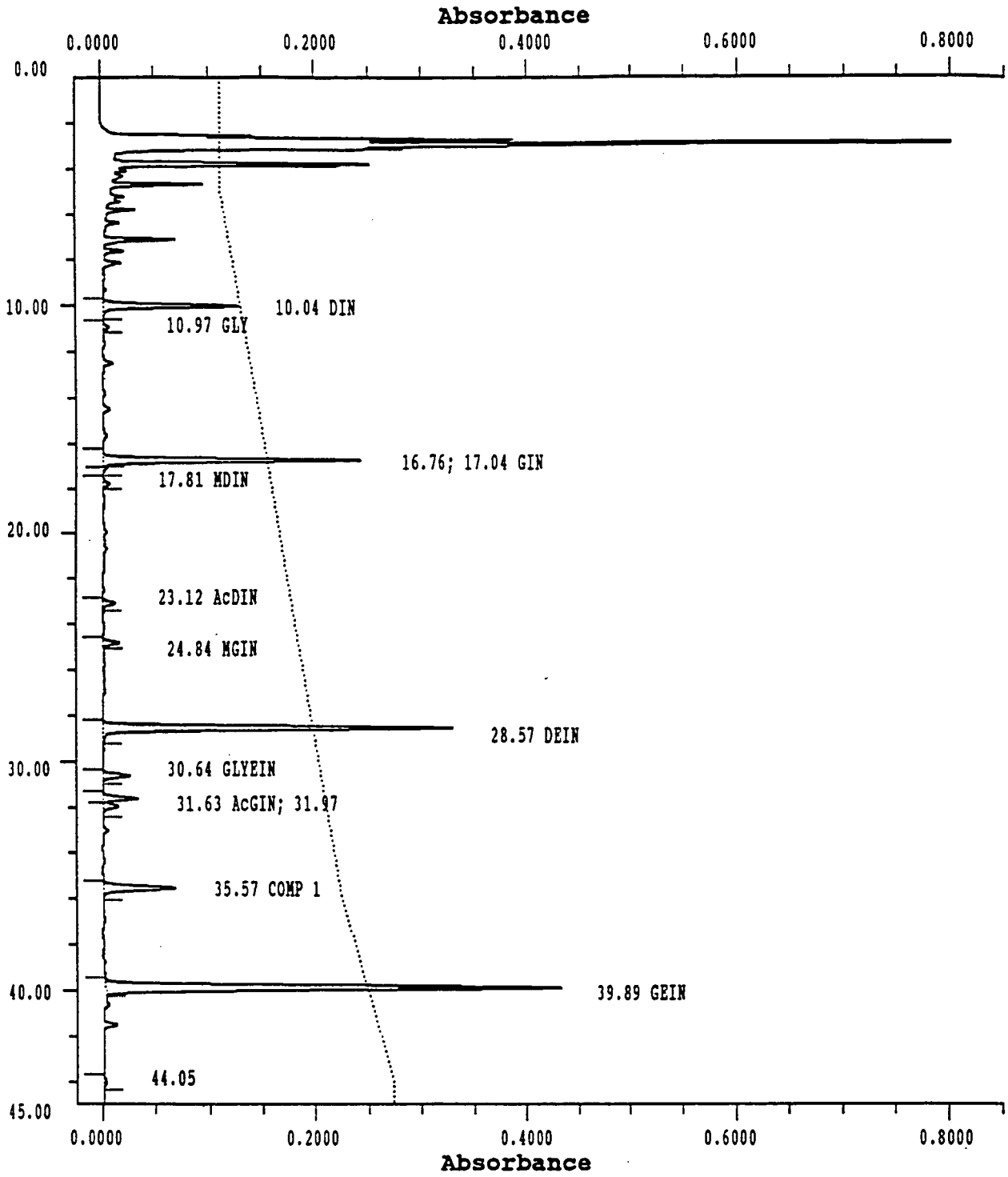

Absorbance
0.0000
0.2000
0.4000
0.6000
0.8000
0.00
10.00
20.00
30.00
40.00
45.00
10.04 DIN
10.97 GLY
16.76; 17.04 GIN
17.81 MDIN
23.12 AcDIN
24.84 MGIN
28.57 DEIN
30.64 GLYEIN
31.63 AcGIN; 31.97
35.57 COMP 1
39.89 GEIN
44.05
Absorbance

Figure 4. Chromatogram of coumestrol standard

NO DATA, CHANNEL A

CHANNEL A INJECT 01:16:96 12:32:47

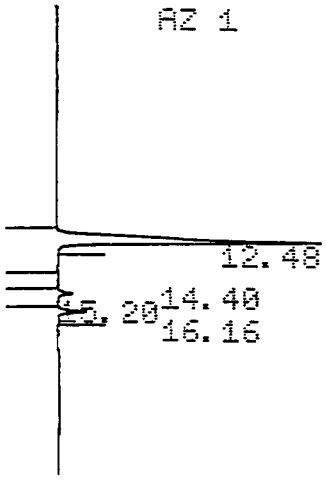


Table 9. Distribution of daidzein compounds (μg/g, as is) in miso[a]

Days of	Daidzin		Daidzein		Acetyl Daidzin		Malonyl Daidzin		Moisture
Fermentation	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	(%)
0	153	7	13	1	42	2	46	10	48.44
1	136	4	23	1	43	5	42	6	46.88
2	129	5	31	2	44	6	44	6	46.78
3	122	2	37	2	38	2	40	8	45.93
4	113	3	41	1	36	2	40	9	45.89
5	107	2	46	1	36	2	40	8	45.57
6	102	4	50	2	38	7	36	8	45.37
8	95	6	63	2	47	16	36	8	45.61
10	83	1	73	2	38	6	31	6	45.69
12	78	2	75	3	37	5	29	6	47.46
15	76	1	85	7	39	2	27	4	47.89
22	75	4	93	4	35	5	23	5	47.70
29	73	5	99	6	31	3	18	4	47.58
36	72	3	98	6	24	3	16	2	47.31
43	73	6	104	3	21	3	13	3	47.23
50	72	6	103	7	21	2	12	2	46.34
57	70	5	101	8	21	3	12	3	45.76
64	70	7	101	4	19	2	10	2	46.78
84	62	6	101	6	15	1	6	1	N/A[b]

[a]Data from three replications.

[b]Not available.

Table 10. Distribution of genistein compounds (μg/g, as is) in miso[a]

Days of	Genistin		Genistein		Acetyl Genistin		Malonyl Genistin	
Fermentation	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
0	208	25	19	1	65	4	59	12
1	164	8	40	2	62	5	58	10
2	165	18	54	4	63	5	58	10
3	142	5	67	4	62	3	57	11
4	136	13	75	2	60	3	57	11
5	128	14	85	2	60	2	55	10
6	122	22	92	3	59	4	55	8
8	104	9	111	3	59	4	53	10
10	90	3	126	1	58	4	46	8
12	90	8	129	4	55	5	42	7
15	91	10	137	4	53	3	40	6
22	89	15	141	5	49	5	34	6
29	89	12	145	2	46	3	29	5
36	93	11	147	7	44	2	25	3
43	97	12	149	5	41	3	19	4
50	105	4	148	7	39	2	18	3
57	99	7	144	13	37	4	18	5
64	97	9	136	8	33	2	14	3
84	88	3	130	6	27	2	9	1

[a]Data from three replications.

Table 11. Distribution of glycitein compounds (μg/g, as is) in miso[a]

Days of Fermentation	Glycitin		Glycitein		Acetyl Glycitin		Malonyl Glycitin	
	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
0	22	2	1	2	4	3	11	1
1	21	2	6	0	0	0	9	1
2	21	2	6	1	2	3	9	1
3	21	2	7	0	2	3	9	1
4	20	2	7	0	0	0	9	2
5	20	2	7	0	6	5	9	1
6	19	2	8	1	4	6	6	5
8	18	4	11	1	2	4	6	5
10	12	1	14	1	4	6	6	4
12	10	1	15	1	3	4	6	4
15	9	1	17	2	3	4	8	0
22	9	2	18	2	1	3	6	4
29	8	1	19	2	0	0	3	4
36	8	1	19	2	6	5	1	3
43	8	1	20	2	6	4	1	3
50	9	1	18	3	4	5	0	0
57	8	1	17	1	0	0	0	0
64	8	2	18	2	0	0	0	0
84	7	0	17	2	0	0	0	0

[a]Data from three replications.

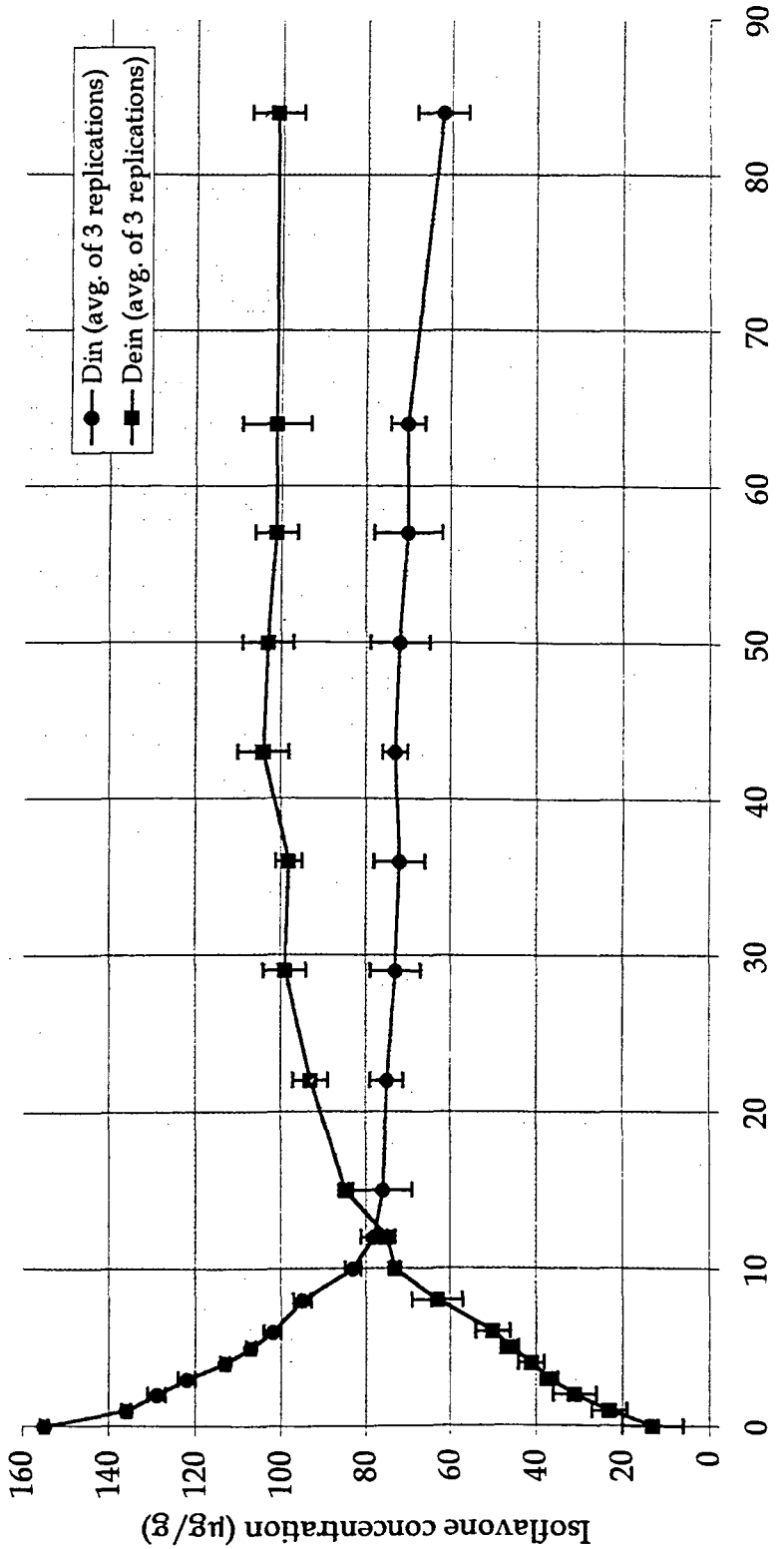


Figure 5. Redistribution of daidzin and daidzein during miso fermentation, due to enzymatic deglycosylation. Samples were analyzed as-is.

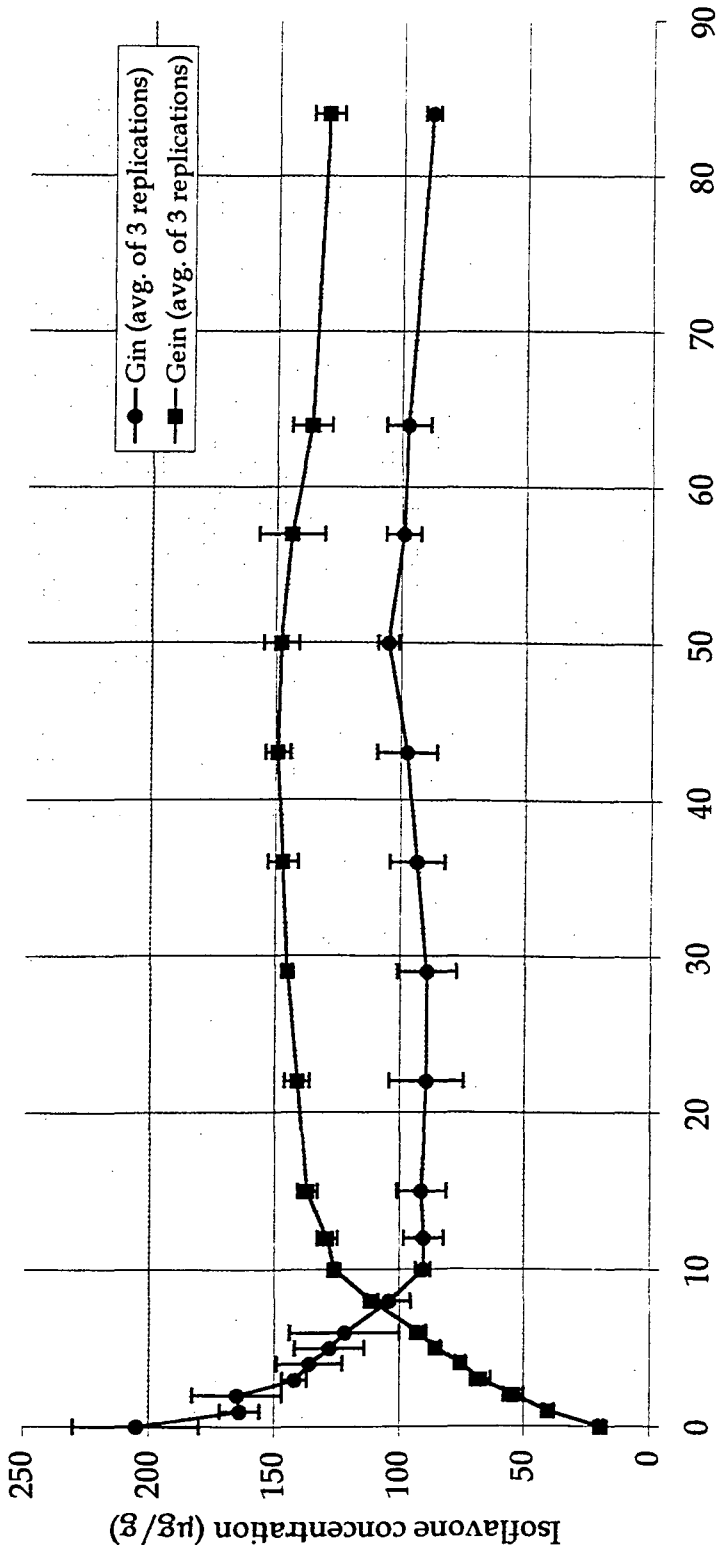


Figure 6. Redistribution of genistin and genistein during miso fermentation, due to enzymatic deglycosylation. Samples were analyzed as-is.

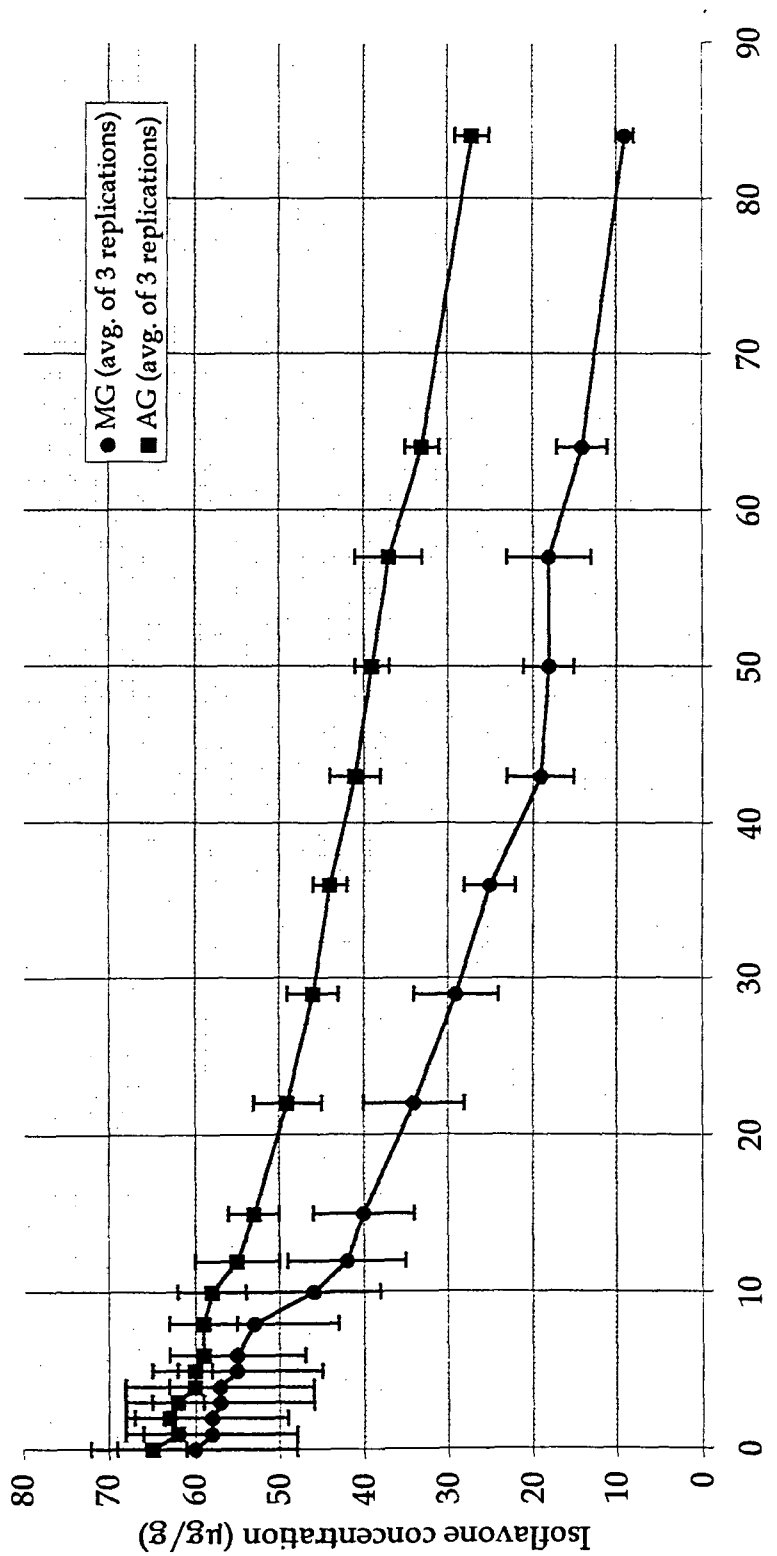


Figure 7. Changes in the acetyl and malonyl forms of genistin during miso fermentation. Samples were analyzed as-is.

Table 12. Normalized and total isoflavones (μg/g, as is) in miso[a]

Days of Fermentation	Total Daidzein Mean	Total Daidzein Standard Deviation	Total Genistein Mean	Total Genistein Standard Deviation	Total Glycitein Mean	Total Glycitein Standard Deviation	Total Isoflavones
0	154[b]	8	216	18	23	5	392
1	151	4	208	7	24	2	383
2	156	4	224	12	26	1	405
3	153	4	221	6	26	4	400
4	151	6	224	8	25	2	399
5	152	4	228	6	28	4	409
6	152	5	230	13	26	7	407
8	165	16	237	6	27	5	429
10	160	8	239	2	27	7	425
12	158	6	239	7	26	6	424
15	168	7	245	8	29	4	442
22	170	6	242	13	27	6	439
29	170	4	243	8	25	3	437
36	163	4	244	13	28	5	435
43	167	4	243	10	29	4	439
50	165	6	246	10	26	5	437
57	161	8	237	21	22	1	420
64	159	8	223	15	23	3	404
84	150	3	205	5	22	2	377

[a]Data from three replications.

[b]Mean total daidzein = 254.23 x [(μg/g Din/416.37) + (μg/g MD/502.41) + (μg/g AD/458.4) + (μg/g Dein/254.23)].

miso would contain if all forms of these isoflavones were hydrolyzed to aglycones.

Figure 5 shows a rapid decline in the concentration of daidzin with a corresponding increase in daidzein concentration. Most of the hydrolysis of glucosides to aglycones has taken place by day 10 or 12. This finding agrees with Ikeda, *et al.* (1995), who reported a decrease in the glucosides daidzin and genistin, with a consequent increase in the aglycones. Figure 6 depicts the same effect for genistin and genistein. The slopes of the changes in the initial and final concentrations of all four of the compounds (din, dein, gin, and gein) was different from 0 ($p < 0.05$).

Microorganisms in the fermentation mixture produce enzymes that hydrolyze bonds and change the forms of the isoflavones. Some enzymes are introduced with the koji, others are formed by the yeast *Z. rouxii,* and others may have been produced by bacteria which were present as salt-tolerant contaminants. One result of the enzymatic activity is the hydrolysis of the ester bond between the isoflavone and the glucose moiety. This explanation of the changes is supported by Ikeda *et al.* (1995), who showed that β-glucosidase activity increased in fermenting Mamemiso and reached a maximum at 12 days, coinciding with the point of maximum concentration of the aglycones. The aglycone concentrations then remained steady through 4 months of fermentation. Analysis of the grits which were autoclaved with

the same water : grits proportion as those prepared for miso shows that the changes of isoflavone forms do not occur to the same extent in the absence of the fermenting microorganisms. Table 13 shows these results.

The Din : Dein and Gin : Gein trends in the experimental miso described here earlier either do not appear in the commercial samples or are much less pronounced. Figure 8 shows that the concentration of din at day 57 is almost unchanged from the initial value at day 0. The slope of the change in din from day 0 to day 57 is not different from 0 ($p < 0.05$). While it may be assumed that an optimum fermentation temperature was provided for the commercial miso, temperature is a possibly relevant point of difference. The jars of miso from which samples were taken in this lab were removed from the incubator for not longer than 10 minutes for sampling. The samples were kept at room temperature (20 - 25° C) for less than 0.5 hour before storage at -29° C. The commercial samples were sent to this lab by unrefrigerated air express and, upon transfer to ground transportation, were subjected to ambient temperatures in an enclosed vehicle in record-breaking summer heat. The temperature may have become high enough to modify enzyme activity. The miso made in this lab was incubated at 31° C, and the fermentation process increases the mixture to a higher internal temperature. Some β-glucosidases have maximum activity at temperatures as high as 60°C (Christakopoulos, *et al.* 1994). Given the fact that the sample of the initial

Table 13. Distribution of isoflavones (μg/g, as is) in miso ingredients and controls[a,b]

Ingredient/ Control	Din	Gly	Gin	MD	MGly	AD	AGly	MG	Dein	Glein	AG	Gein
Soybeans	158	47	171	1321	131	0	0	1424	20	0	28	20
Soybean hulls	123	38	308	58	0	0	0	221	24	0	0	64
Soybean grits, cooked	282	39	325	100	16	67	7	103	15	2	100	20
Miyako miso used as starter	67	7	99	17	0	20	7	29	95	19	45	134
Control grits, day 1	328	46	379	33	4	94	3	39	15	5	137	20
Control grits, day 5	321	43	403	43	12	90	10	38	14	5	132	20

[a]All soybeans, hulls, and grits are from the same lot of Vinton 81 variety.

[b]Data from three replications.

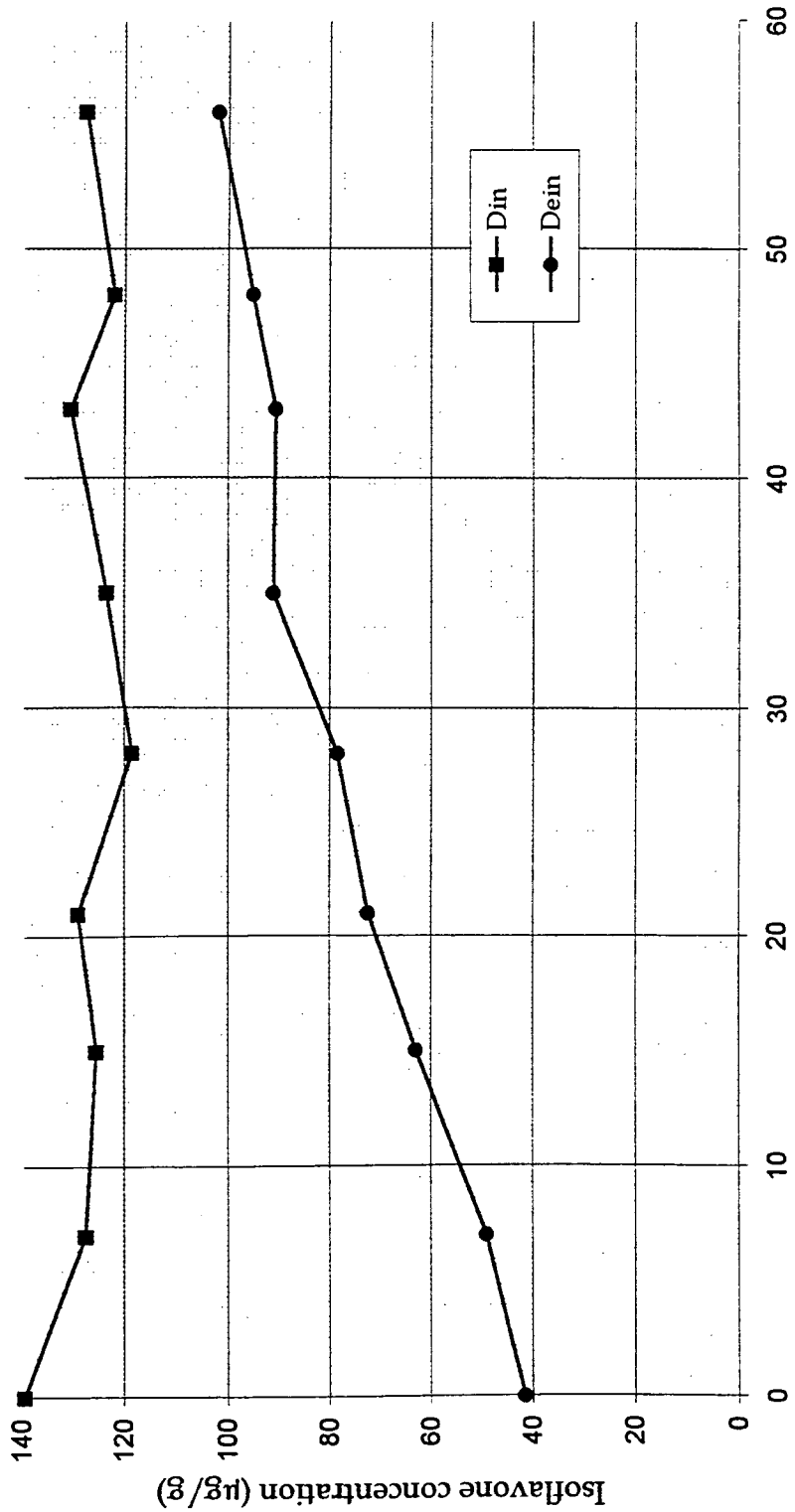


Figure 8. Redistribution of daidzin and daidzein in commercial miso during fermentation. Values are from one replication.

fermentation mixture from the commercial source was prepared on July 6, 1995, and received here on July 10, 1995, after travel in conditions which almost certainly exceeded 31°C, and the likelihood that the rate of hydrolysis of the glucose substituent was accelerated, it is quite possible that the Din and Dein values plotted at day 0 are more closely equivalent to the values for about day 10 in Figure 5. This likelihood is further supported by the fact that the samples received on July 10 were refrigerated but not frozen until a few days after their arrival, allowing additional time for enzymatic activity, although it would have been at a reduced level. The same pattern is shown with the trends of Gin and Gein in the commercial miso (see Figure 9) compared to Figure 6.

The difference in rates of hydrolysis of Din to Dein does not appear to be explained by differences in the initial Din and Dein content of the soybeans. The (Din : Dein) ratio for the soybeans used by the commercial miso maker was (7.8 : 1), compared to (7.6 : 1) for the soy grits used to make miso here. There is a difference in the (gin : gein) ratios, with soybeans for the commercial miso being (2.1 : 1) compared to (8.1 : 1) for soy grits used here, although the trends in Figures 8 and 9 are very similar. Other unknown differences in the soybeans may also be involved, as well as differences in the fermenting microorganisms.

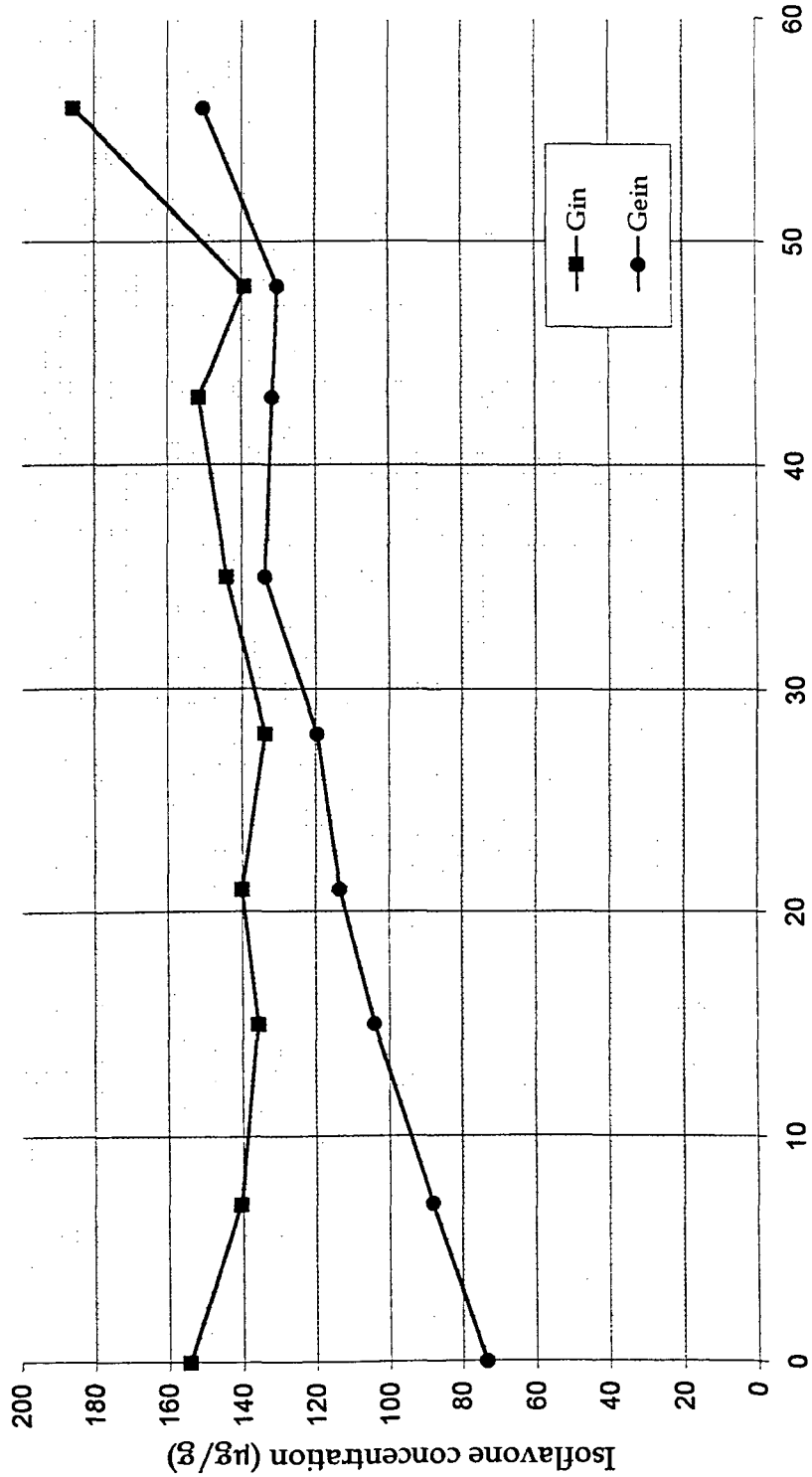


Figure 9. Redistribution of genistin and genistein during commercial miso fermentation. Values are from one replication.

Figure 7 shows that the acetyl and malonyl forms of genistin both decline steadily over the course of the fermentation. These acetyl and malonyl findings agree very closely with the analyses of the commercial miso samples, the results of which are shown in Table 14 and Figure 10. Heating converts malonyl forms of the isoflavones to glucosides and, eventually, the acetyl-substituted forms. The heat produced by the fermenting mixture is adequate to cause this reaction, as is ambient heat during storage or transportation. It is therefore reasonable that the variations in temperature which could explain the discrepancy in the Din and Dein trends previously discussed reinforces the lack of a discrepancy between Figures 10 and 7.

Total isoflavone concentrations in miso, normalized by adjusting the molecular weights to those of the aglycone forms (see Table 12), are fairly constant. Calculation of the mass balance (Tables 15 and 16) demonstrates that, while the isoflavones are changing forms, isoflavones were neither produced nor lost over 57 days of fermentation.

The fermentation of tempeh can be used as a model to help understand the isoflavone changes which are taking place in miso. Tempeh is a soy, or rice and soy food product which is also inoculated with a mold. As described earlier in this paper, research has demonstrated that hydrolysis of the glucosides Din and Gin to Dein and Gein occurs early. The entire fermentation period is only 1 to 3 days (Wuryani, 1995). According to Wuryani, the hydrolysis rate of glucosides to aglycones depends on the rate of production

Table 14. Distribution of isoflavones (μg/g, as is) in commercial miso during fermentation[a]

Days of Fermentation	Din	Gly	Gin	MD	MGly	AD	AGly	MG	Dein	Glein	AG	Gein
0	140	20	155	43	8	47	0	61	42	0	74	74
7	128	19	141	38	8	43	0	55	49	6	69	88
15	126	17	136	34	8	37	0	50	63	8	67	104
21	129	14	140	32	4	36	0	47	73	12	65	114
28	119	12	134	25	0	30	0	38	79	14	59	120
35	124	10	144	22	0	29	0	35	91	15	60	134
43	131	11	152	18	0	27	0	27	91	15	58	132
48	122	9	139	17	0	26	0	26	95	16	54	130
56	128	11	186	12	0	25	0	22	102	17	54	151

[a]Data from one fermentation.

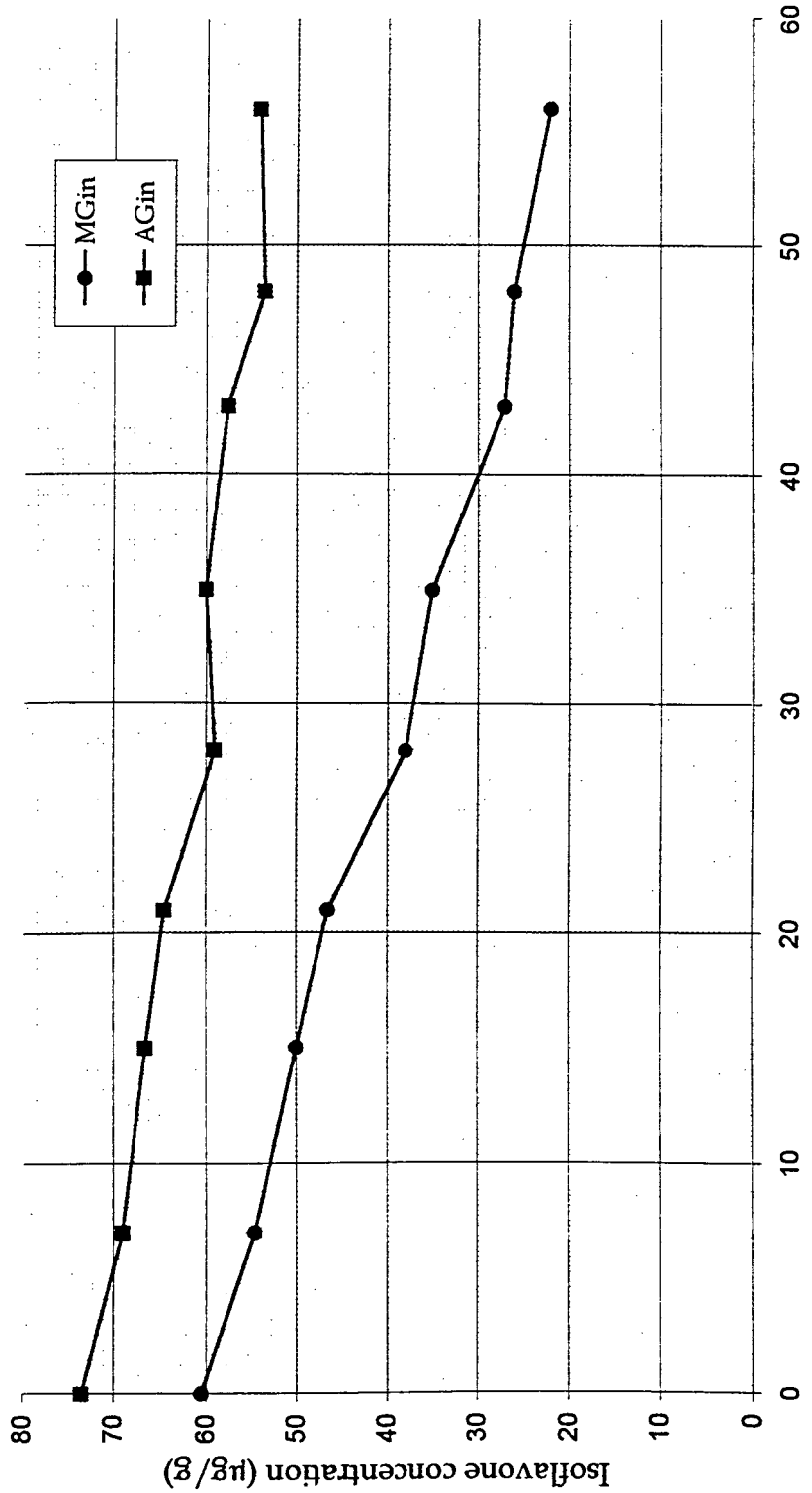


Figure 10. Changes in the acetyl and malonyl forms of genistin during commercial miso fermentation. Values are from one replication.

Table 15. Normalized and total isoflavones (μg/g, as is) in miso ingredients and controls[a,b]

Sample Type	Total Daidzein Mean	Total Daidzein Standard Deviation	Total Genistein Mean	Total Genistein Standard Deviation	Total Glycitein Mean	Total Glycitein Standard Deviation	Total Isoflavones
Soybeans	785	11	885	16	99	2	1769
Soybean hulls	129	19	372	35	24	1	525
Soy grits, raw	756	75	884	67	71	17	1710
Soy grits, cooked	274	5	334	2	40	7	648
Starter miso	155	2	237	8	28	4	419
Control grits, day 0	280	7	359	4	32	10	671
Control grits, day 1	284	15	355	12	38	10	677
Control grits, day 5	281	8	366	27	44	6	691

[a]All soybeans, hulls, and grits are from the same lot of Vinton 81 variety.

[b]Data from three replications.

Table 16. Mass balance of isoflavones in fermenting miso

Procedure and Materials	Yield (g)	Moisture[a] (%)	Total Isoflavones[b] (μg/g)	Total Isoflavones[c] (mg)
Soy grits preparation				
Soybeans	626	10.70	1769± 28.59	1107± 17.90a
Hulls and fines	126	12.55	59± 55.67	7.4± 7.0
Soy grits	500	10.72	1710± 159.04	855± 79.5 c
Miso preparation				
Soy grits	500		1710± 159.04	855± 79.5 c
Soaking and cooking water	1000		0	0
Miso starter	50	45.32	419± 14.40	21± 0.72
Other ingredients	890		0	0
Miso fermentation				
Initial mixture	2440	48.67	392± 31.54	956± 77.0 b
Miso at day 1	2440[d]	45.67	420± 29.80	1024± 72.7 b
Miso at day 57	2100[e]	45.67	420± 29.80	882± 62.6 bc

[a]Moisture is calculated as the difference in sample weights before and after freeze-drying.

[b]Measured by HPLC, on an as is basis.

[c]Calculated as: Total Isoflavones (mg/batch) = Yield (g) x Total Isoflavones (μg/g).

[d]Assumes the final weight equals the beginning weight.

[e]Estimated final weight, assumes a 20g sample removed at each of 17 sampling times.

Values in columns with different letters were significantly different at α=0.05.

of β-glucosidase by *Rhizopus* and also varies according to the species of *Rhizopus*.

Soybean sprouts

Figure 11 shows the pattern of coumestrol formation in the sprouting soybean seeds over 7 days of growth. Figures 12 through 15 show trends in isoflavone content over the same time period. The apparent increases in concentration of Din and Dein may be partially explained by the loss of other, more water soluble mass during watering of the sprouts. Since daidzein has been shown to be a precursor of coumestrol (Hahlbrock and Grisebach, 1975, p. 906), a Dein decrease rather than an increase may be expected if the Dein was being consumed as substrate for the increase in coumestrol. Table 21 is a mass balance of isoflavones in sprouting soybeans from 0 through 168 hours of growth. The concentrations of total normalized isoflavones at 0 hours and at 24 hours are different ($p < 0.05$), but there is no difference in the total at 24 hours compared to all other harvest times.

Isoflavone and coumestrol intake

Isoflavone research to date shows biological activity for genistein and daidzein (Lu *et al.*, 1995; Xu *et al.*, 1994). Also, research indicates that glucoside forms of isoflavones are hydrolyzed to aglycones by intestinal microflora

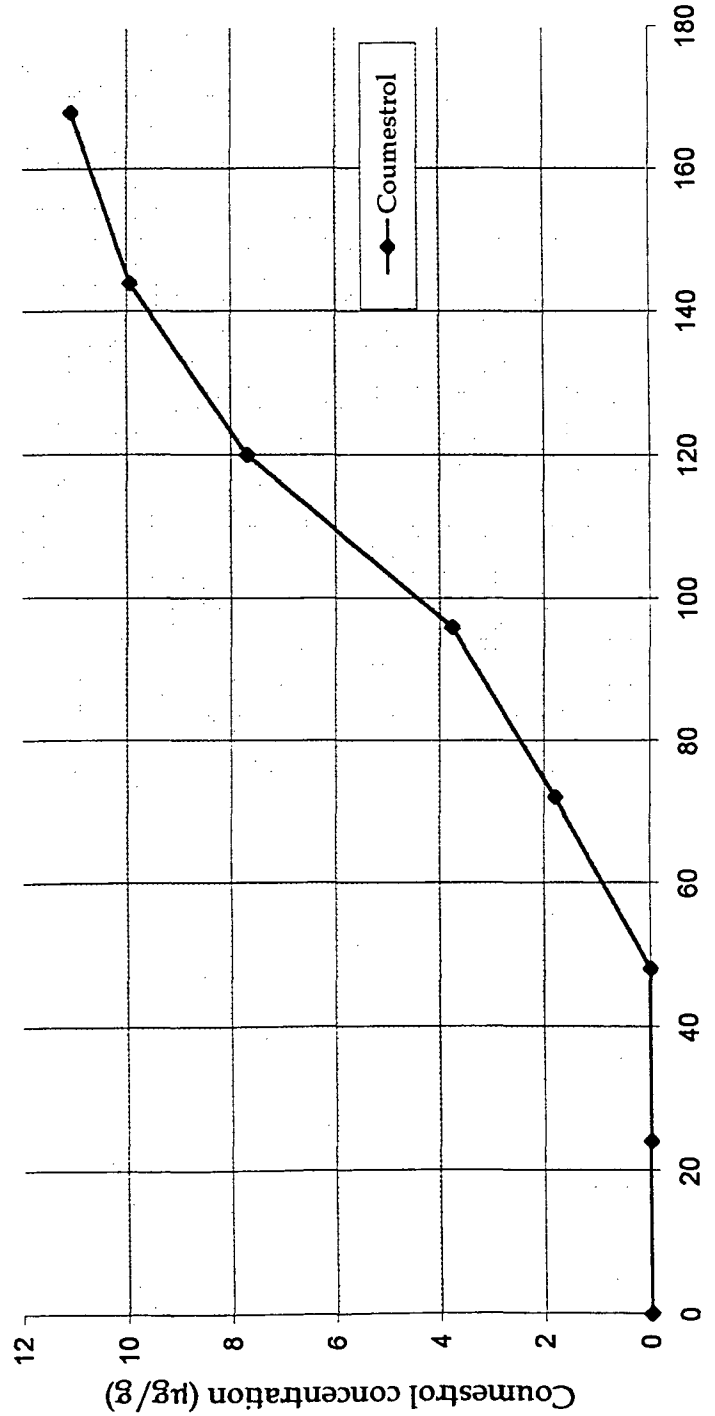


Figure 11. Formation of coumestrol in sprouting soybean seeds (µg/g, freeze-dried). Values are the averages of three replications.

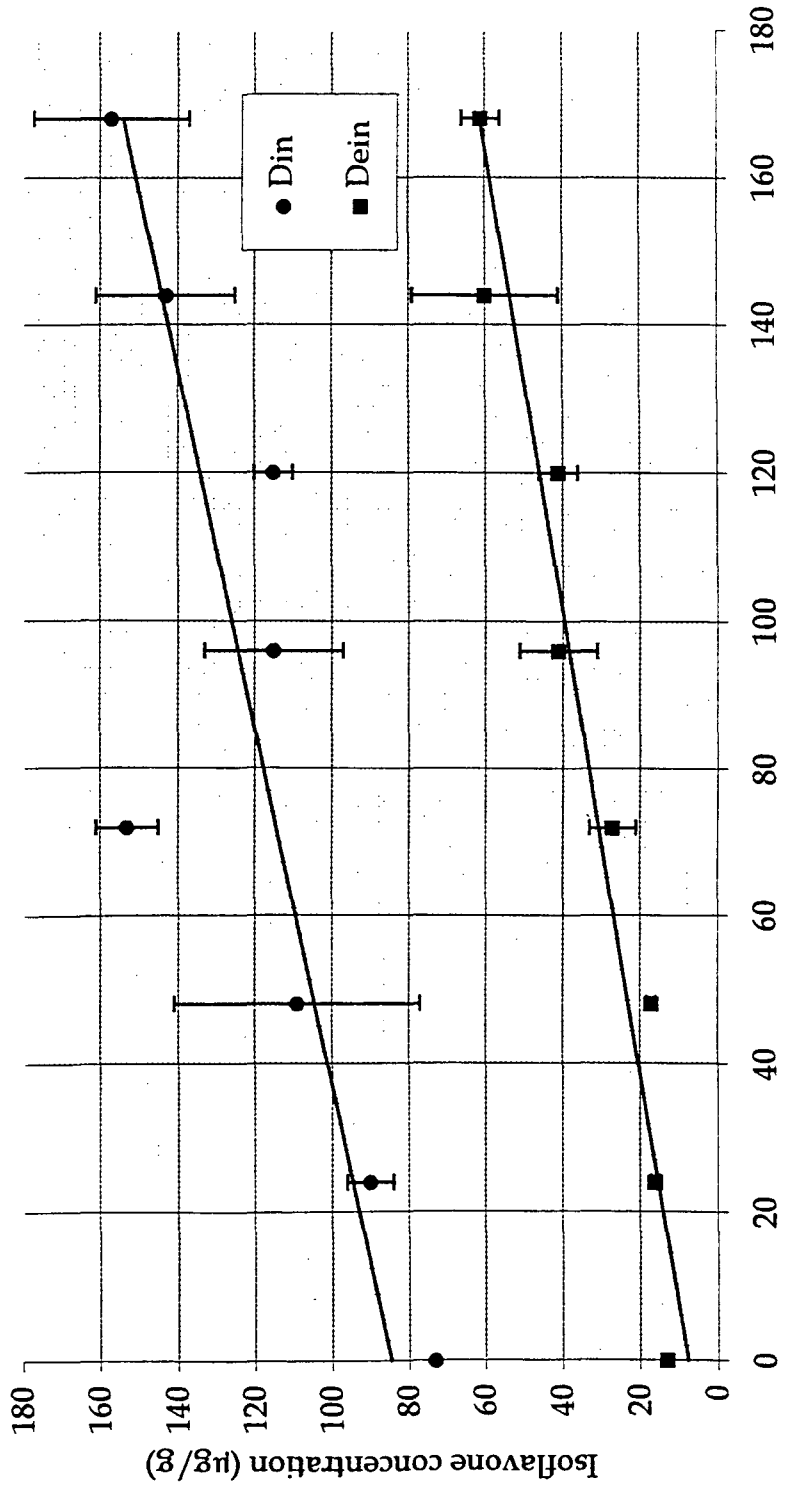


Figure 12. Changes in concentration of daidzin and daidzein in sprouting soybean seeds (µg/g, freeze-dried). Values are averages of three replications.

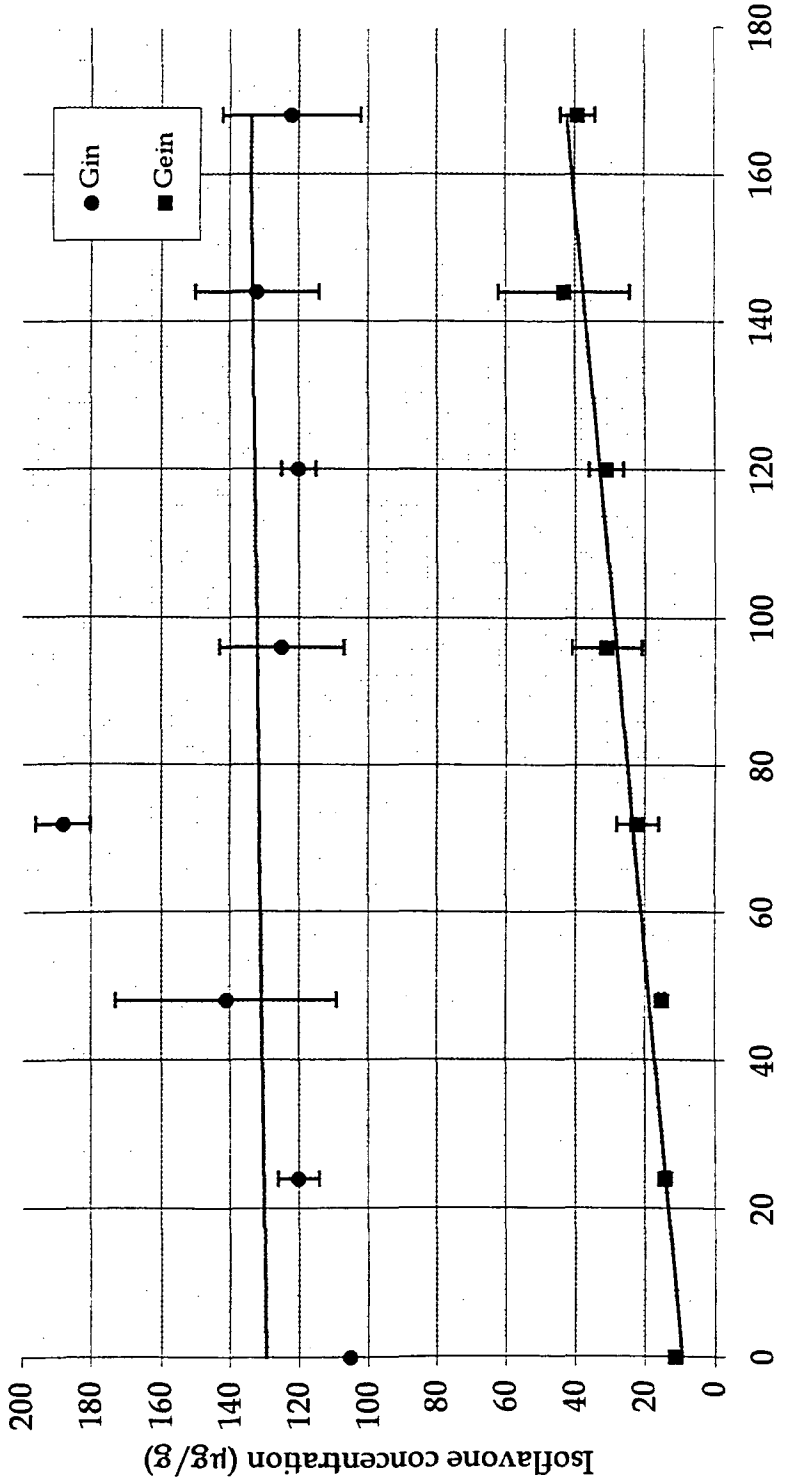


Figure 13. Changes in concentration of genistin and genistein in sprouting soybean seeds (µg/g, freeze-dried). Values are averages of three replications.

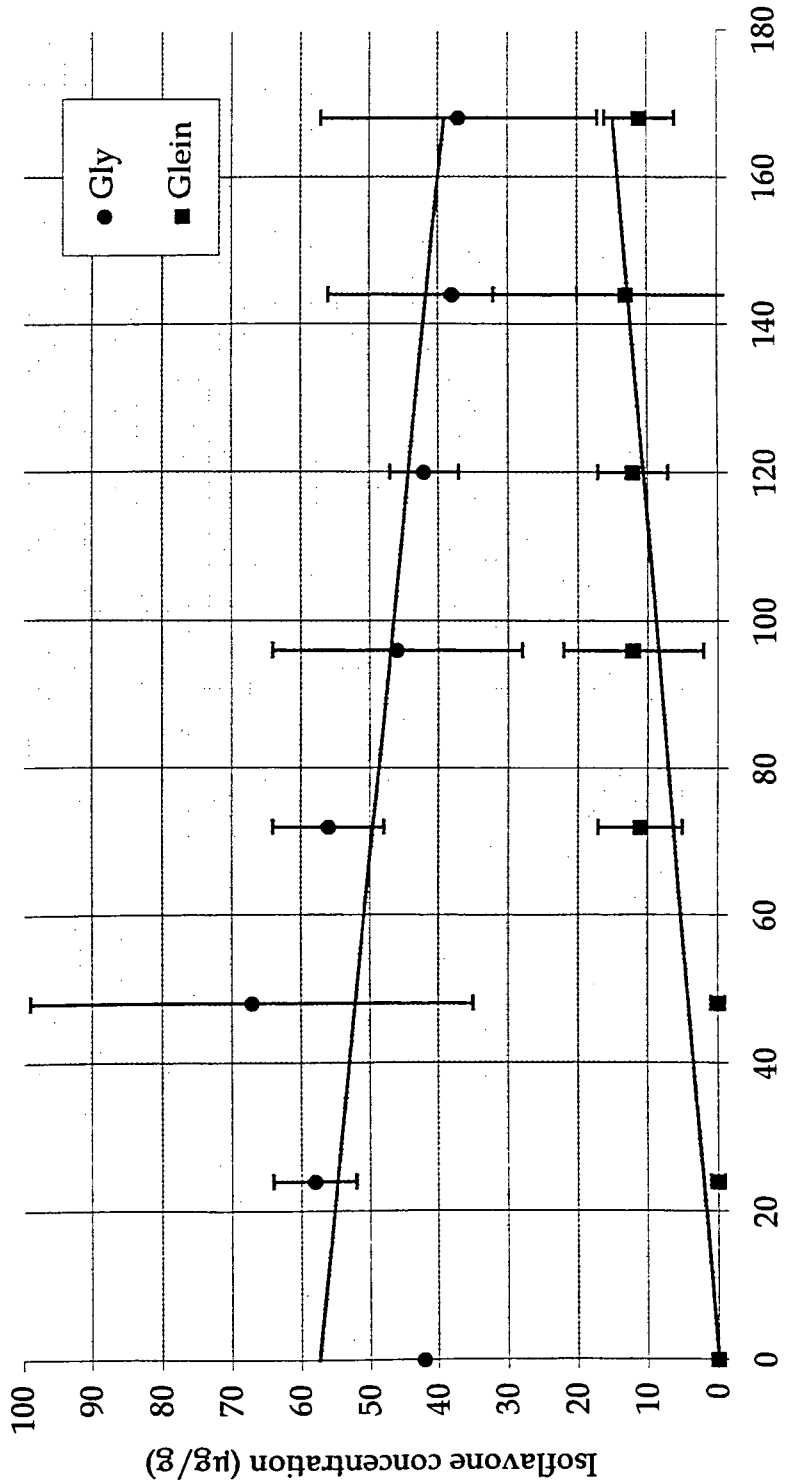


Figure 14. Changes in concentration of glycitin and glycitein in sprouting soybean seeds (µg/g, freeze-dried). Values are averages of three replications.

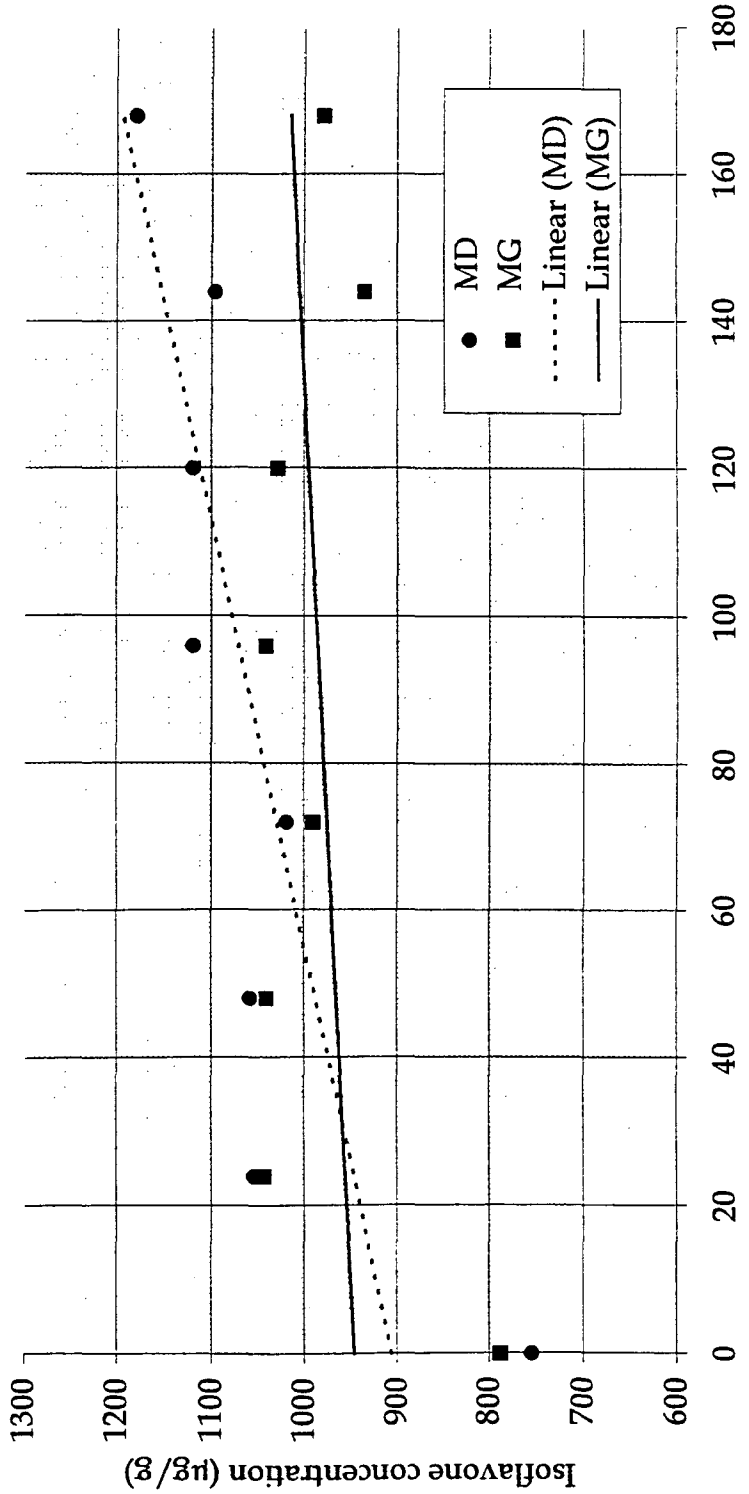


Figure 15. Changes in concentration of the malonyl forms of daidzin and genistin in soybean sprouts (µg/g, freeze-dried). Values are averages of three replications.

Table 17. Distribution of daidzein compounds (μg/g, freeze dried) in sprouting soybean seeds[a]

Hours of	Daidzin		Daidzein		Acetyl Daidzin		Malonyl Daidzin		Moisture[b]
Growth	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	(%)
0	73	N/A[c]	13	N/A	0	0	755	N/A	15
24	90	6	16	1	0	0	1054	45	48.72
48	109	32	17	1	0	0	1058	69	50.31
72	153	8	27	6	0	0	1019	53	64.56
96	115	18	41	10	0	0	1119	82	72.68
120	115	5	41	5	0	0	1119	20	71.27
144	143	18	60	19	0	0	1096	155	73.90
168	157	20	61	5	0	0	1180	9	78.65

[a]Values for hours 24 - 168 are based on 3 replications; values for hour 0 beans are from one analysis.

[b]Moisture was determined by near infrared spectroscopy for hour 0 beans; all others were measured as the difference in weights before and after freeze-drying.

[c]N/A = Not available.

Table 18. Distribution of genistein compounds (μg/g, freeze-dried) in sprouting soybean seeds[a,b]

Hours of	Genistin		Genistein		Acetyl Genistin		Malonyl Genistin	
Growth	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
0	105	N/A[c]	11	N/A	0	N/A	788	N/A
24	120	5	14	0	0	0	1043	8
48	141	43	15	1	0	0	1040	66
72	188	10	22	5	0	0	990	36
96	125	24	31	6	0	0	1041	61
120	120	3	31	4	0	0	1028	5
144	132	9	43	17	0	0	935	55
168	122	7	39	8	0	0	979	63

[a]Values for hours 24 - 168 are based on 3 replications; values for hour 0 beans are from one analysis.

[b]Moisture was determined by near infrared spectroscopy for hour 0 beans; all others were measured as the difference in weights before and after freeze-drying.

[c]N/A = Not available.

Table 19. Distribution of glycitein compounds (μg/g, freeze-dried) in sprouting soybean seeds[a,b]

Hours of	Glycitin		Glycitein		Acetyl Glycitin		Malonyl Glycitin	
Growth	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
0	42	N/A[c]	0	N/A	0	N/A	124	N/A
24	58	8	0	0	0	0	166	22
48	67	7	0	0	0	0	169	10
72	56	12	11	2	0	0	165	2
96	46	6	12	1	0	0	193	32
120	42	1	12	0	0	0	213	12
144	38	1	13	2	0	0	236	5
168	37	6	11	1	0	0	217	9

[a]Values for hours 24 - 168 are based on 3 replications; values for hour 0 beans are from one analysis.

[b]Moisture was determined by near infrared spectroscopy for hour 0 beans; all others were measured as the difference in weights before and after freeze-drying.

[c]N/A = Not available.

Table 20. Normalized and total isoflavones and coumestrol, (μg/g, freeze-dried) in sprouting soybean seeds[a,b]

Hours of Growth	Total Daidzein[c]		Total Genistein		Total Glycitein		Total Isoflavones	Coumestrol
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation		
0	440	N/A[d]	488	N/A	93	N/A	1021	0
24	605	27	633	1	126	16	1364	0
48	619	15	645	17	133	4	1397	0
72	636	24	656	18	135	6	1427	1.79
96	677	36	651	24	144	20	1472	3.76
120	677	17	642	6	152	7	1472	7.70
144	702	71	613	17	163	1	1478	9.93
168	754	101	626	23	151	9	1531	11.1

[a]Values for hours 24 - 168 are based on 3 replications; values for hour 0 beans are from one analysis.

[b]Moisture was determined by near infrared spectroscopy for hour 0 beans; all others were measured as the difference in weights before and after freeze-drying.

[c]Total daidzein = 254.23 x [(μg/g Din/416.37) + (μg/g MD/502.41) + (μg/g AD/458.4) + (μg/g Dein/254.23)]

[d]N/A = Not available.

Table 21. Mass balance of isoflavones in sprouting soybean seeds[a]

Hours of Growth	Single Batch Wt. (g)	Moisture (%)[b]	Normalized Total Isoflavones (μg/g)	Normalized Total Isof., as-is, (μg/g)	Normalized Total Isof. (mg/batch)
0	120.0 ± 0.1	15	1021	868	104 a
24	224.1 ± 2.4	48.7	1364 ± 44	700	157 ± 31 b
48	232.5 ± 4.3	50.3	1397 ± 36	694	161 ± 14 b
72	316.7 ± 10.0	64.6	1427 ± 48	506	160 ± 24 b
96	359.6± 18.5	72.7	1472 ± 80	402	161 ± 32 b
120	382.9 ± 5.4	71.3	1472 ± 30	423	162 ± 13 b
144	438.3 ± 14.4	73.9	1478 ± 89	386	170 ± 34 b
168	505.9 ± 25.0	78.7	1531 ± 133	327	165 ± 43 b

[a]Values are averages of 3 replications.

[b]Moisture was determined by near infrared spectroscopy for hour 0 beans; all others were measured as the difference in weights before and after freeze-drying.

Values in columns with different letters were significantly different at α=0.05.

prior to absorption (Adlercreutz *et al.*, 1995). Therefore, in estimating intake of isoflavones, it is reasonable to look at total normalized Dein and Gein. A simple single serving of miso soup made with 2 teaspoons of light yellow miso fermented for this study (assuming 4.3 g per teaspoon) and one-half cup of hot water, according to Table 12, would contain 3.44 mg of Dein and Gein. According to Messina (1995), Japanese daily intake of isoflavones can be estimated at 60 - 90 mg/day, based on estimates of per capita soybean intake.

Cassidy *et al.* (1995) determined that premenopausal women experienced physiological effects from the consumption of 50 g/day of miso containing 25 mg of unconjugated isoflavones. Specifically, the women experienced a mean menstrual cycle length increase of 5 days, but the sample was too small to test for statistical significance.

Based on the total Dein, total Gein, and coumestrol content of soybean sprouts from Table 20, a person who ate 1 cup of soybean sprouts (approximately 105 g raw) would consume 31 mg of the isoflavones and a little more than 1 mg of coumestrol. Since the focus of coumestrol research has been on livestock and not humans, very little is known about the significance of that intake of coumestrol from sprouts.

CONCLUSIONS AND RECOMMENDATIONS

The purpose of this research was to add to the base of knowledge regarding the effects of processing on the content and distribution of coumestrol and 12 forms of isoflavones in soy foods. Specifically, these studies present more complete time courses for the fermentation of miso and the sprouting of soybeans than were previously available in the published literature. Twelve forms of isoflavones were quantitated in both products, whereas previous researchers have analyzed only aglycone forms and glucosides and daidzein and genistein.

A small laboratory scale miso method was used successfully to produce miso which was comparable to a commercial product in appearance, aroma, and flavor. The effect of fermentation on the isoflavone content and distribution of light-yellow miso was demonstrated, with support from analyses of commercial samples and published data. In general, glucosides were hydrolyzed to aglycones by about day 10, and acetyl and malonyl forms declined steadily throughout the fermentation. A mass balance showed no net change in total isoflavones with the miso method used in the study.

Commercial soybean sprout growing methods were successfully applied to a laboratory situation. Coumestrol formed gradually over 168 hours of

growth. Total isoflavones appeared to increase, but this may be a result of loss of water soluble mass during the frequent waterings. Capturing this water and quantitating the isoflavones was not practical due to the large volume of water used.

Future research could include the following points:

- Ferment miso from "typical miso soybeans" and compare the results to miso made from Vinton 81 soybeans.
- Grow sprouts from soybeans used by commercial growers and compare the results to Dekalb 291 soybeans.
- Grow soybean sprouts in an ethylene-enriched atmosphere and compare to sprouts grown in a non-enriched atmosphere.
- Analyze soybean sprouts as they would normally be prepared for consumption, i. e., dehulled, steamed, etc.
- Do sensory testing of the miso and the soybean sprouts.

APPENDIX

Figure A-1. Ultraviolet scan of daidzin

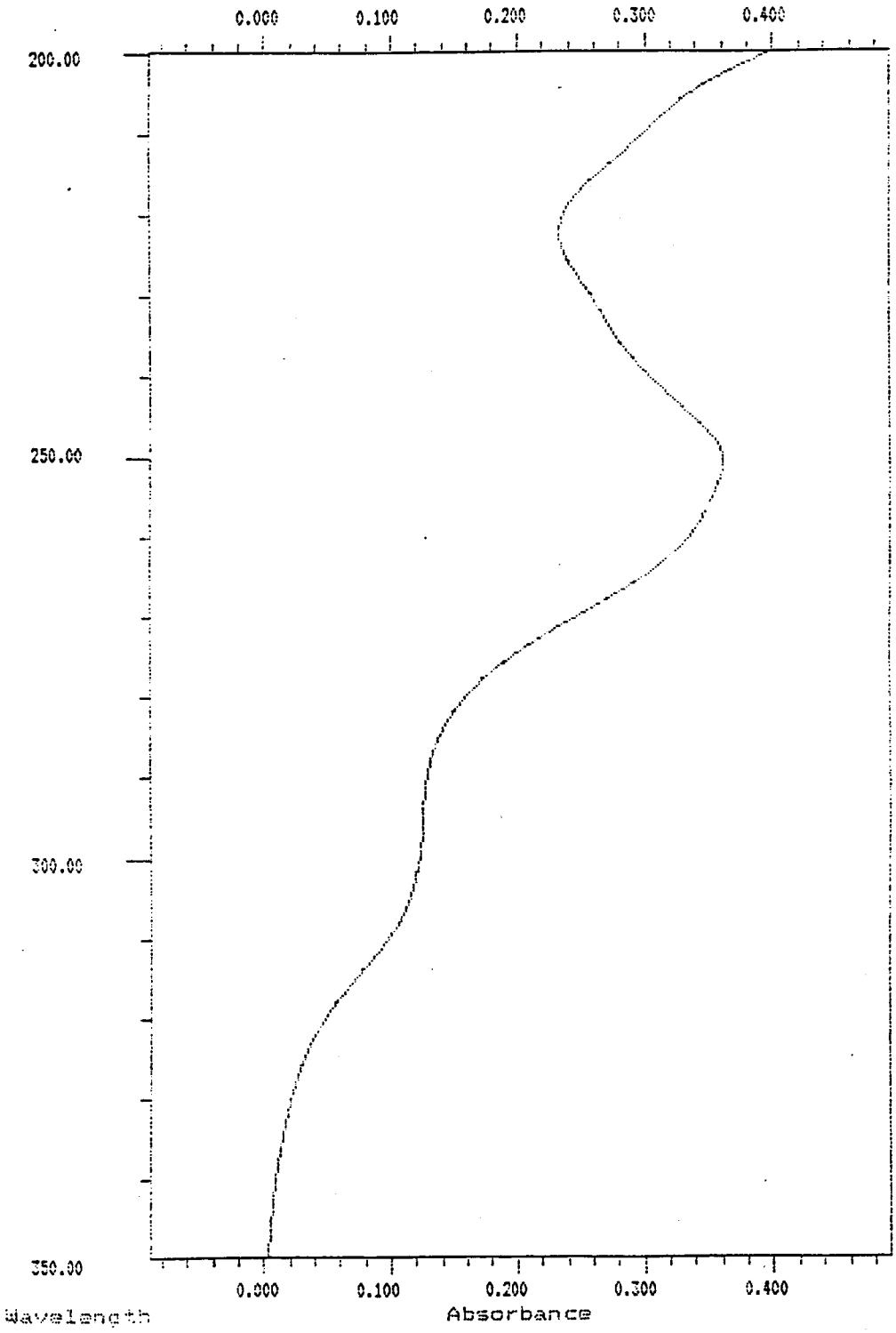

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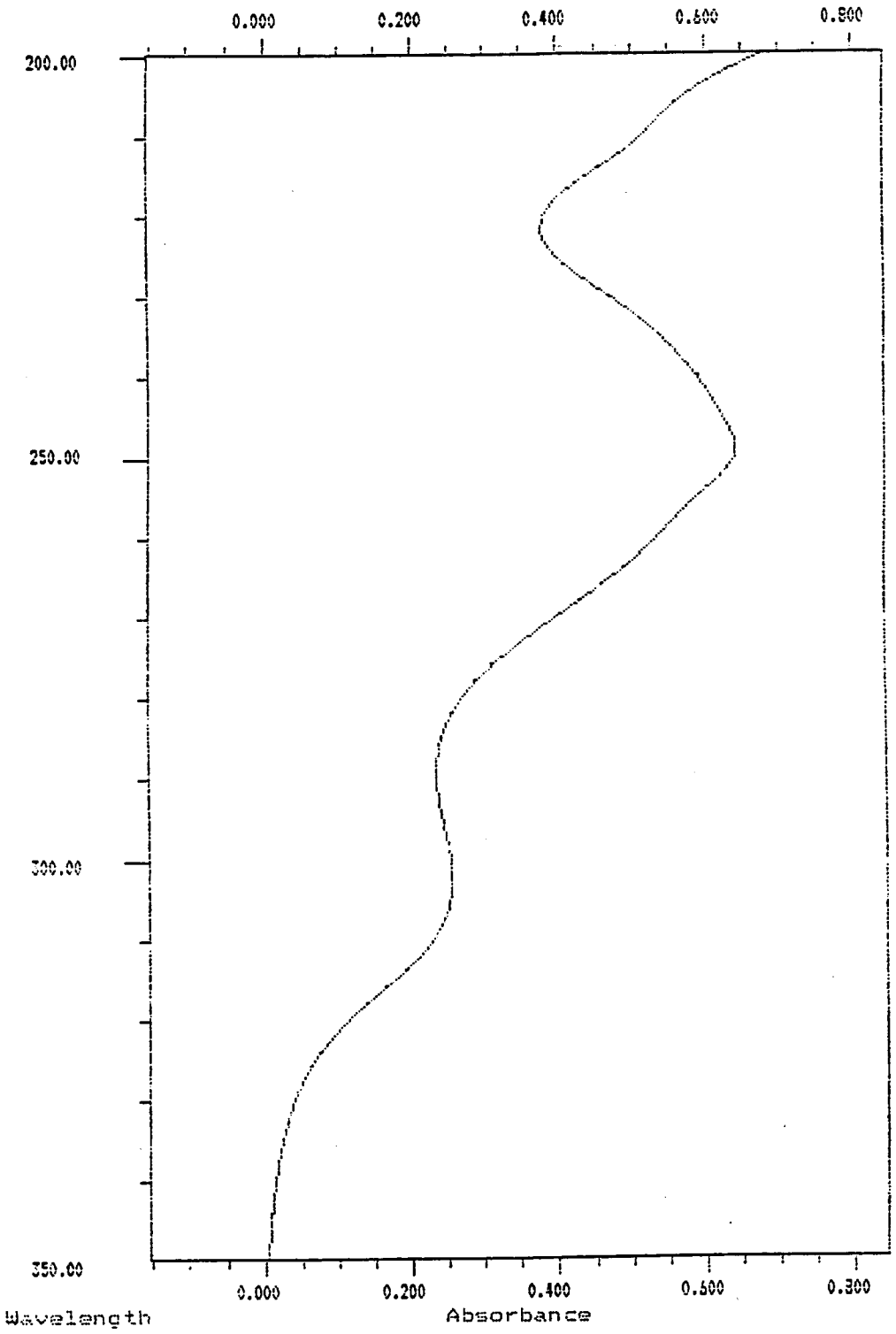
Figure A-2. Ultraviolet scan of daidzein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Wavelength
Absorbance

Figure A-3. Ultraviolet scan of genistin

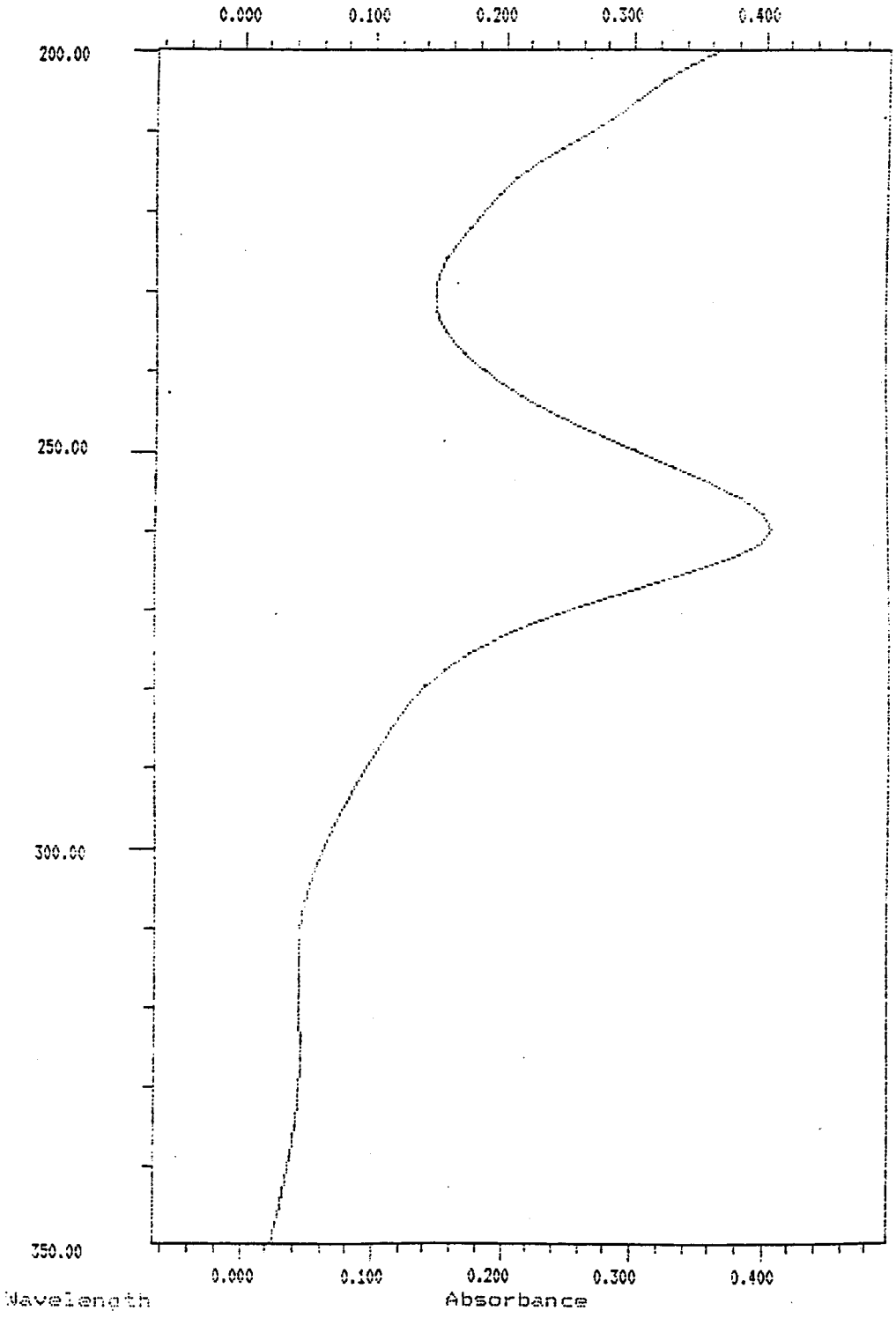
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Figure A-4. Ultraviolet scan of genistein

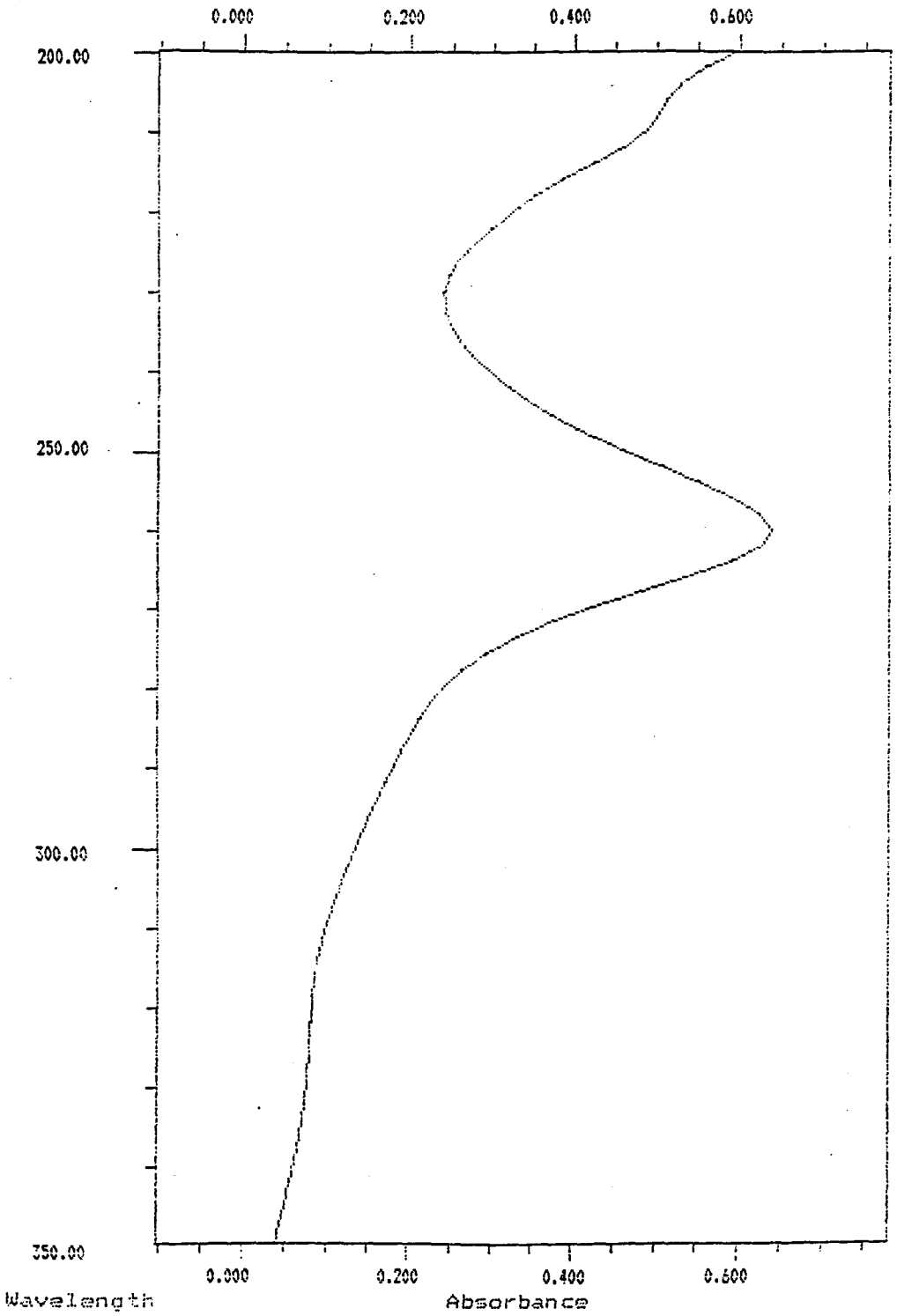
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Figure A-5. Ultraviolet scan of glycitin

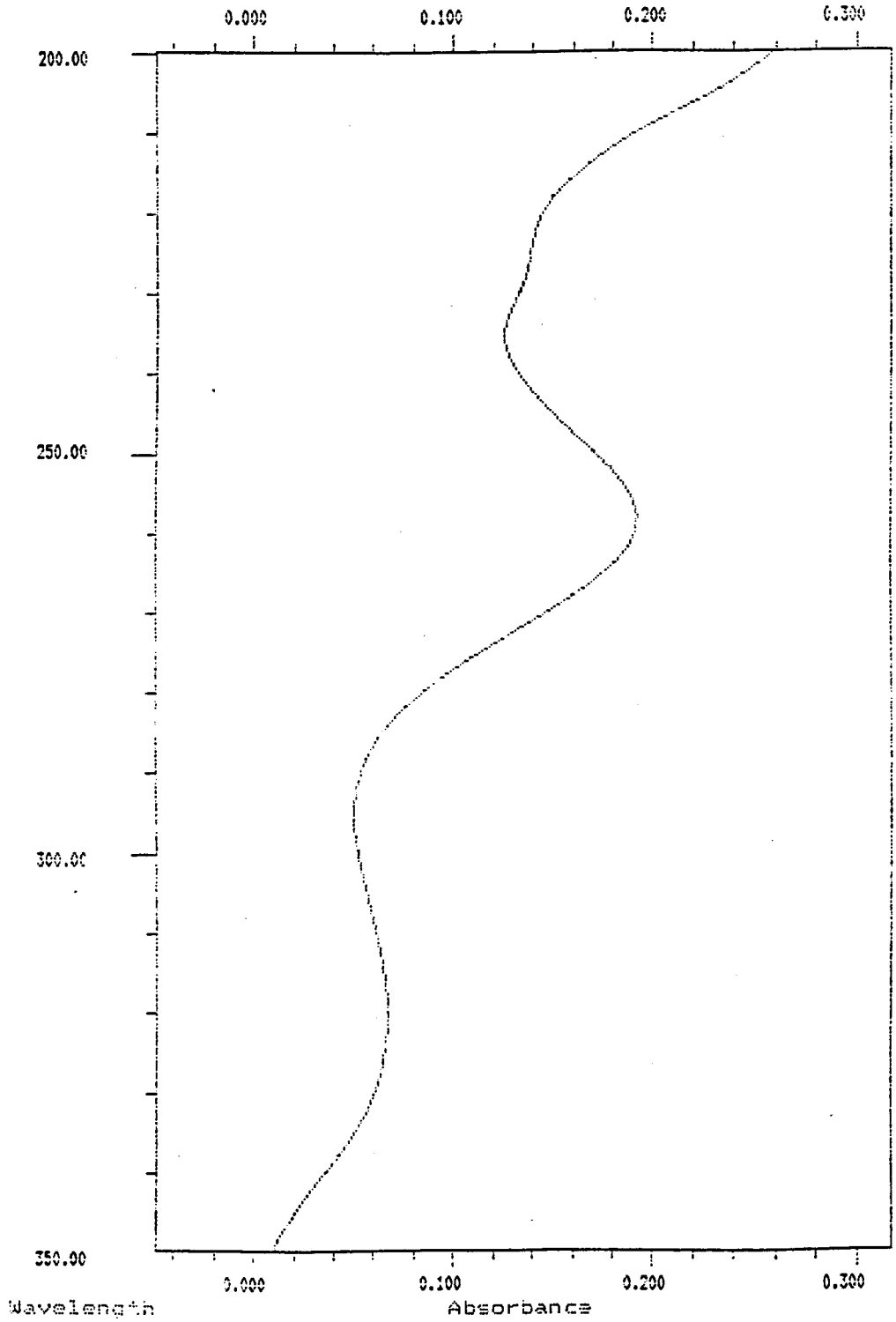
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Figure A-6. Ultraviolet scan of glycitein

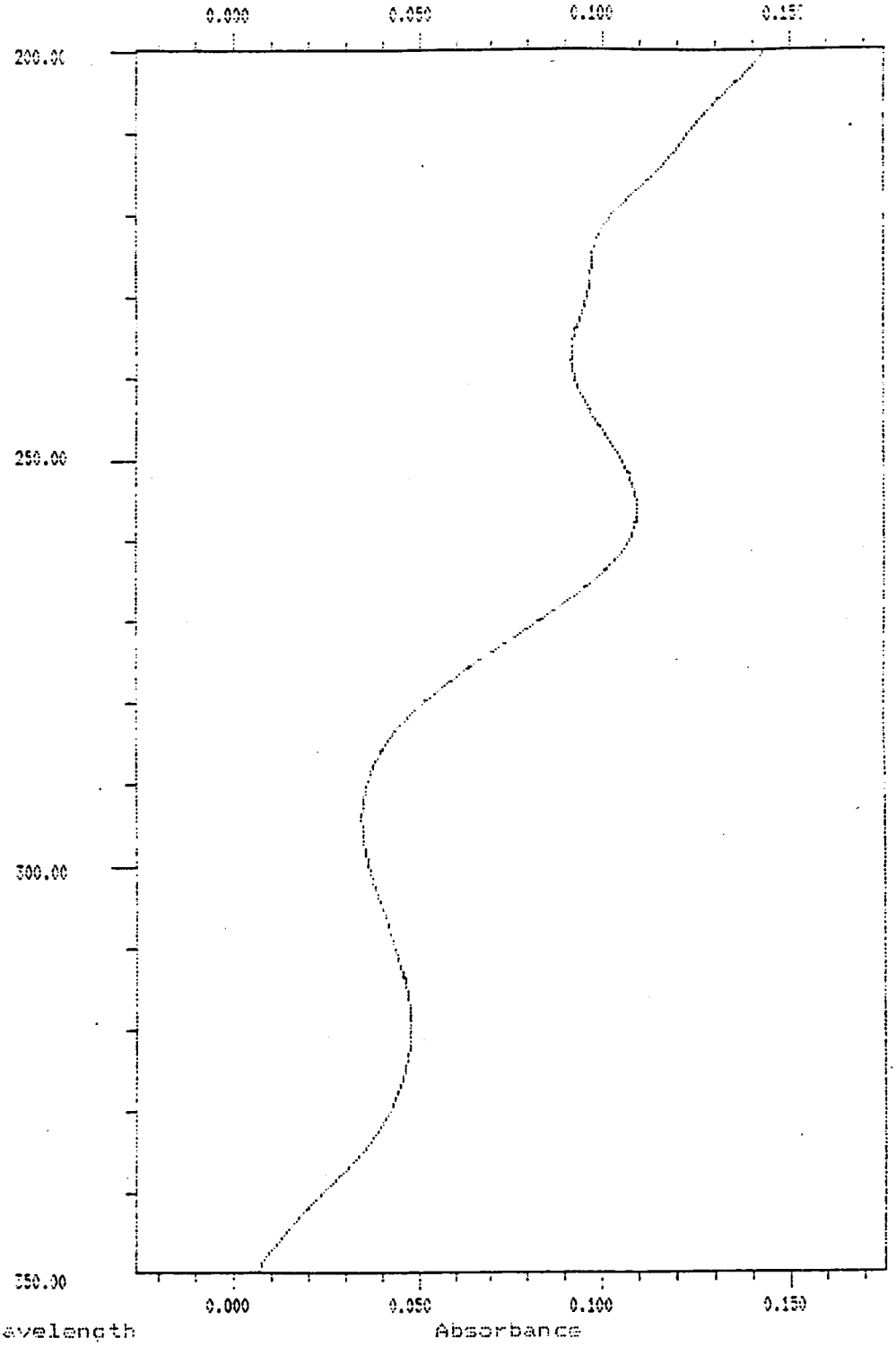
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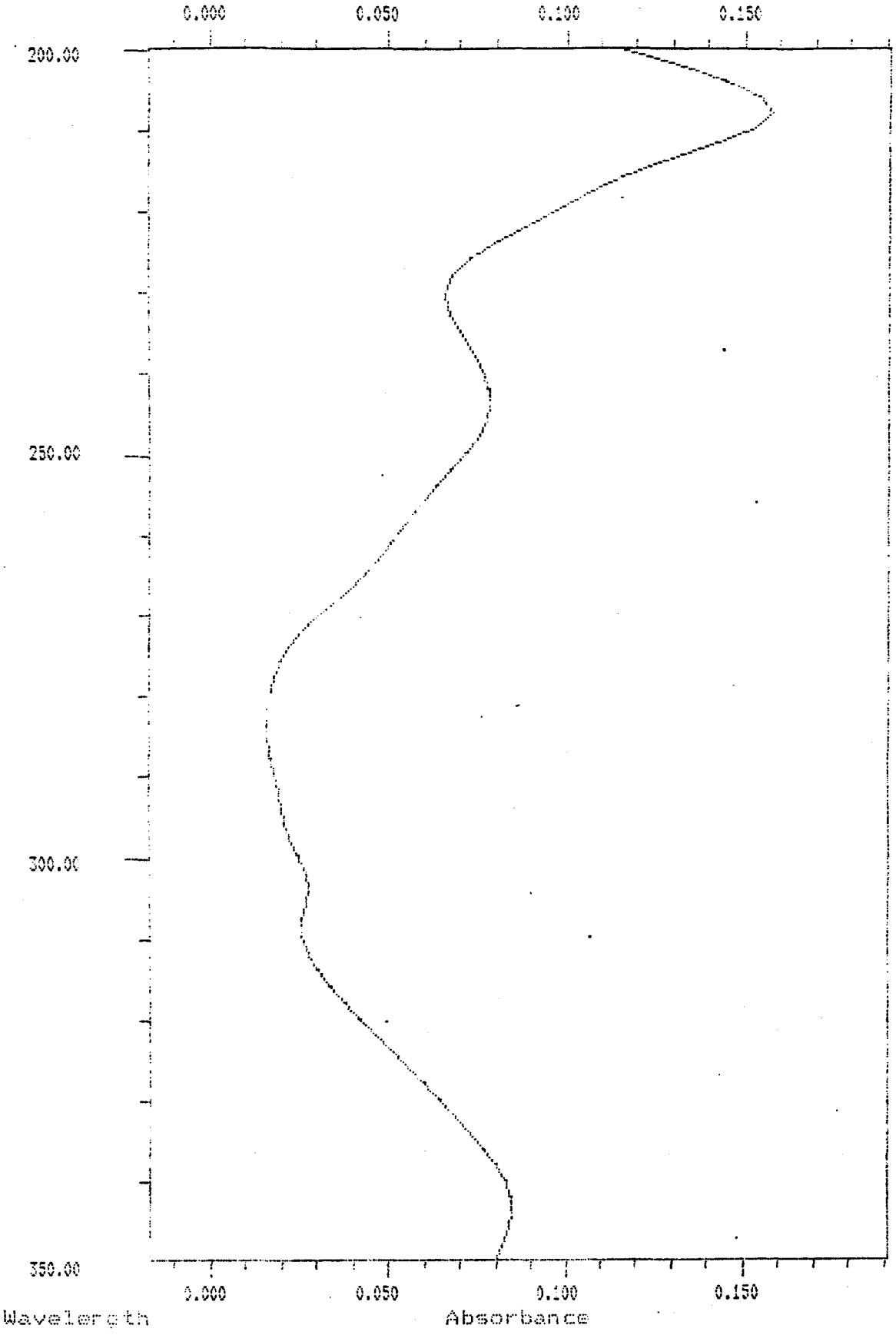
Figure A-7. Ultraviolet scan of coumestrol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