THE EFFECT OF VARIOUS ACIDIC SOLUTIONS ON THE CONCENTRATION OF GENISTEIN IN TEMPEH

by

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ABSTRACT

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Tempeh is a fermented soyfood that contains a higher level of genistein in comparison to nonfermented soyfoods, such as tofu and soymilk. Control tempehs prepared in this research contained concentrations of genistein seven times greater (49.70 mg/g dry sample) than that of unprocessed soy grits (7.08 mg/g dry sample).

The isoflavone genistein is of interest because of its potential anticarcinogenic effect. The main objective of this project was to investigate the effects that various acid solutions had on the concentration of genistein in tempeh. Lactic acid, citric acid, and acetic acid (0.1% solutions) were used in the soaking and/or cooking periods.

The concentration of genistein in tempeh samples was measured by HPLC. Genistein concentrations in the tempehs made by methods involving acidic soaking and/or cooking solutions ranged from 24.83 ± 7.69 to 46.33 ± 7.47 mg/g dry sample. Control tempehs, prepared from soy grits soaked in water for1 hour and cooked in water, had genistein concentrations of 49.70 ± 3.64 mg/g dry sample. Based on the data collected in this project, the use of an acidic solution in the soaking period and/or the cooking period did not enhance the genistein concentration in tempeh.

Continuing research in our laboratory is being conducted to explore the use of a ß-glucosidase enzyme that when added to the soaking solution has the potential to hydrolyze the glucose molecule attached to genistin, thus forming genistein.

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CHAPTER I

INTRODUCTION

The United States of America was known as the healthiest country in the world primarily due to the fact that "the sick stayed home and the weak died on the way." This is, of course, in regard to the immigrants coming to America in order to search for a better life with religious freedoms and democracy. Today, this better life consists of high employment rates, governmental-funded programs, technologically-advanced medical care, and an immense variety of processed foods. Despite all of the advantages found in the United States, significant health problems exists in America. The American Cancer Society predicts that 1,222,100 new cases of cancer will be diagnosed in the United States in 2000 and that 552,200 Americans will die of cancer in 2000.[1] According to the World Health Organization's compilation of cancer data from 45 countries around the world collected between the years 1994-1997, the incidence of cancer in females in the United States was ranked 7th in the world whereas China was ranked 37th and Japan was ranked 42nd.[1] The United States is ranked 14th in female breast cancer incidence whereas Japan was ranked 43rd and China was ranked 44th.[1]

Differences in the incidence of cancer in Asian and American cultures may be due to fundamental differences in diet. Americans consume a typical Western diet consisting of high-fat foods while those in Asian cultures typically consume a low-fat diet. Consumption of soybean products, such as tofu, miso, and tempeh, as well as low-fat foods is common in Asian cultures. Among the Japanese, the incidence of cancer is less for Japanese living in Japan compared to Japanese who have immigrated to Hawaii from Japan.[2] That difference may reflect differences in the diet. Diet and cancer risk

may be associated with the amount of soy products consumed by individuals and the incorporation of more soybean products into the American diet may decrease cancer risk.[3,4]

When women reach menopause, biological changes occur that cause an increased incidence of chronic diseases like osteoporosis.[5] As a result, scientists are conducting research to develop treatments that will protect women from the effect of hormonal imbalances that accompany menopause. In addition to the pharmacological research for the treatment of the aforementioned diseases, many researchers are focusing on the benefits provided through the diet. The prevention of chronic disease by dietary manipulation would reduce the amount of money spent on medical procedures necessary once the onset of a chronic diseases has been diagnosed. In addition to dietary modifications introduced to decrease disease risk, lifestyle changes can improve the quality of one's health and prevent or slow the onset of diseases.[6] Cessation of smoking, the incorporation of heart rate-increasing exercise routines, and the addition of a weight-lifting regimen can improve body composition by bringing about weight reduction, an increase in muscle mass, and easing breathing difficulties.[7]

The medical community is taking a more proactive approach to health than ever before, attempting to prevent disease or delay its onset rather than just treat existing disease. Dietary and lifestyle changes are well-established ways to help prevent disease.[6] In order to help Americans choose a healthy diet, the United States Department of Agriculture (USDA) developed the Food Guide Pyramid in 1992.[7] The Food Guide Pyramid provides a suggested number of servings for each division or category of food. Figure 1 depicts the USDA Food Guide Pyramid.[7]

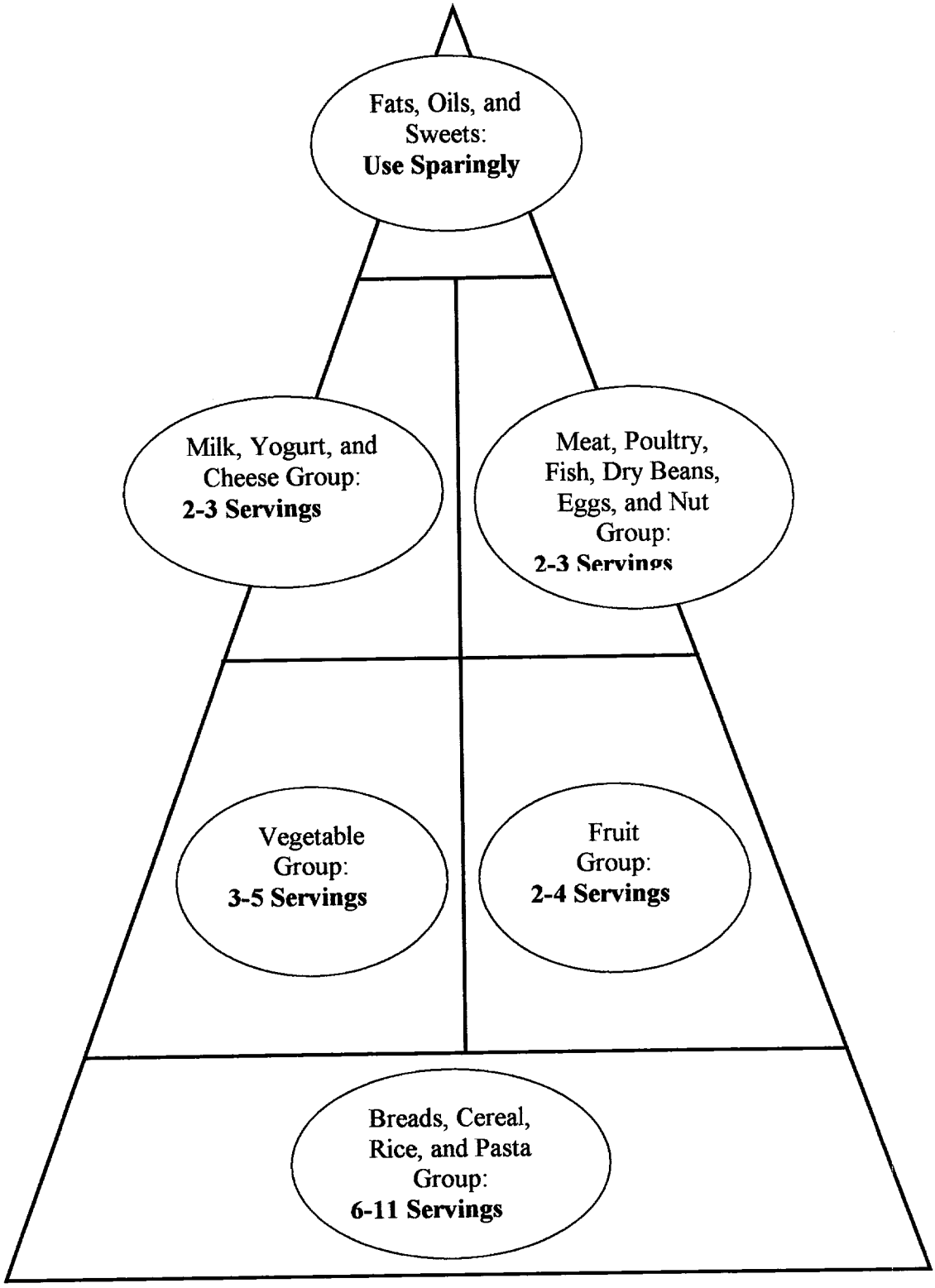


Figure 1. United States Department of Agriculture's Food Guide Pyramid[7]

In addition to the Food Guide Pyramid, many published guidelines exist that suggest ways for Americans to develop a balanced diet and healthy lifestyle practices. In 1995, for example, the United States Department of Agriculture and the United States Department of Health and Human Services published Dietary Guidelines for Americans in an effort to prevent or delay the onset of chronic disease.[7] There are seven guidelines:

- Eat a variety of foods.
- Balance the food you eat with physical activity; maintain or improve your weight.
- Choose a diet with plenty of grain products, vegetables, and fruit.
- Choose a diet low in fat, saturated fat, and cholesterol.
- Choose a diet moderate in sugars.
- Choose a diet moderate in salt and sodium.
- If you drink alcoholic beverages, do so in moderation.

In the effort to use diet as a means to curb the onset of disease, one focus has been on the potential health benefits of soybeans and soy products.[8] The focus on soybeans may seem odd, but research on the potential health benefits of the soybean portion of the Asian diet indicates probable health benefits associated with such a diet.[2,8,9] The consumption of soybean products, such as tofu, miso, and tempeh, is common in Asian cultures.[10] In association with increased consumption of soy foods, the incidence of breast, colon, corpus uterine, and prostate cancers is lower for those in the Asian cultures compared to Americans.[2] The incidence of breast cancer is higher in the United States in comparison to Japan.[2]

Soy is a main ingredient in many foods. At times, it is difficult to associate soy products with soybeans. Soy milk, oil, and meat substitutes are all products of soybeans. Most people do not realize that soy sauce, which is frequently added to chow mien or stir-fry, is actually extracted from fermented soybeans. In contrast, soy is commonly associated with soybean oil, a product included in many processed foods and used as a cooking oil.

During the last 10 years, much research has been conducted on the potential benefits of soy-based foods in preventing chronic diseases such as cancer and osteoporosis.[3,4,5,11] These studies often focus on specific substances called isoflavones, which are found in relatively high amounts in soybeans.[11] Common forms of soybean isoflavones have a glucose molecule bonded to the chemical structure. In their nonglucoside form, isoflavones are chemically similar to the human hormone estrogen.

One of the most interesting soybean isoflavones is genistein. Recent findings suggest the maximum health benefits of genistein are evident when it is made available in its nonglucoside form.[12-22] A review of the literature shows that a high level of genistein is present in tempeh made from soybeans.[23-25] Studies have also shown that the methods used to process soybeans to manufacture tempeh may increase the levels of genistein in the final tempeh product.[25-29] Therefore, the research hypothesis for this study is that changing the processes used to manufacture tempeh will increase the level of genistein present in tempeh.

Statement of Problem

The main objective of this study is to evaluate the effects of selected changes in processes used to produce tempeh on the concentration of genistein in tempeh as measured by High Performance Liquid Chromatograph (HPLC). The specific aims for this study are the following:

1. To determine the effects of soaking dehulled soybean grits in various acid solutions or in water on the concentration of genistein in tempeh.
2. To determine the effects of soaking dehulled soybean grits for different lengths of time in various acid solutions or in water on the concentration of genistein in tempeh.
3. To determine the effects of cooking the soaked dehulled soybean grits in various acid solutions on the concentration of genistein in tempeh.

CHAPTER II

REVIEW OF LITERATURE

Dietary modifications, such as the inclusion of soybeans in the diet, are used to prevent or delay the onset of chronic disease like cancer rather than to treat the disease after its diagnosis. Both the American Journal of Clinical Nutrition in 1998 and the Journal of Nutrition in 1995 have published supplemental issues devoted solely to research involving soybean composition, consumption, and its associated health effects.[30,31]

The purported active dietary component in soybeans, the isoflavone genistein, is of particular interest in cancer research.[2] Research interest in the soybean isoflavone genistein and its glucoside form genistin has grown in recent years. It is reported that genistein, the aglycone form, is more effective on health than its aglycone form, genistin.[12] However, genistein is not as abundant in soybeans as genistin.[24,26,27] If genistin could be converted to genistein, then such processed soybeans could be more effective when incorporated into the diet to prevent, delay, or treat chronic diseases like breast cancer.

Soybean Harvest

Commercial processing of soybeans began in the United States in 1922.[32] The cultivation and consumption of soybeans, however, likely originated in Eastern Asia well before written documentation.[32] Today, varieties and strains of soybeans number into the thousands.[32]

The United States Department of Agriculture conducts a Census of Agriculture, which includes soybean production information every five years.[33] The 1997 Census of

Agriculture reported that 66.1 million acres of crop land were used to harvest soybeans, which was second only to crop land used for corn, Table I.[33] According to the USDA 1997 Census of Agriculture, Iowa used 9.9 million acres to harvest soybean, while Illinois used 9.8 million acres and Minnesota used 6.2 million acres, Table II.[33] In 1998, the United States Department of Agriculture reported that the United States produced 48% of the world's soybeans, 75.0 million metric tons (2,757 million bushels).[34]

Table I

Acres Used to Harvest Crops in the United States[33]

Crop	Millions of Acres
Corn for grain	69.8
Soybeans for beans	66.1
Hay – all types	60.8
Wheat for grain	58.8
Cotton	13.2
Sorghum for grain	8.5
Barley for grain	5.9

Source: United States Department of Agriculture: 1997 Census of Agriculture: Selected Crops Harvested: 1997

Table II

Acres Used to Harvest Soybeans for Beans in Seven Mid-west States[33]

State	Millions of Acres
Iowa	9.9
Illinois	9.8
Minnesota	6.2
Missouri	4.7
Nebraska	3.3
South Dakota	2.9
North Dakota	1.1
Wisconsin	1.0

Source: United States Department of Agriculture: 1997 Census of Agriculture: Selected Crops Harvested: 1997

Soybean Composition

Woodruff and Klaas published a chemical analysis of the composition of the soybean, Table III.[35] Table III presents information on the average composition of

soybeans.[35] According to the information presented in Table III, protein comprises 40.6% of the composition of the mature dry soybean. Based on the data in Table III, one pound of mature dry soybeans contains approximately 200 grams of protein, 36 gram of fat, and 152 grams of carbohydrates. Based on 4 calories/gram protein, 9 calories/gram fat, and 4 calories/gram carbohydrate, the majority of the calories in soybeans come from protein.

Table III
Composition of the Mature Dry Soybean[35]

Soybean Unit	Composition
Moisture	7.0 %
Protein (Nitrogen x 6.25)	40.6 %
Fat	16.5 %
Total Carbohydrates	30.9 %
Ash	5.0 %
Calcium	0.212 %
Iron	0.0103 %
Calories per pound	1973

Source: Woodruff and Klaas.[35]

Soybean Protein

Soybeans are known as a quality source of protein.[35] Proteins are linear polymers of various combinations of amino acids. Of the 20 amino acids used in the synthesis of proteins, eight are essential amino acids and 12 are nonessential amino acids, Table IV.[36] The 20 essential and nonessential amino acids are found in sufficient quantity in many animal products but not in many plant-based products.[37] The proteins of soybeans contain adequate amounts of all of the essential amino acids but one, methionine.[9] Although the limiting amino acid in soybeans is methionine, soybeans have a high level of the essential amino acid lysine.[38] All of the necessary amino acids can be obtained in

adequate amounts if soybeans are used in protein complementation, a practice in which consumption of two or more plant protein sources together supply all essential amino acids in sufficient amounts for maintenance and growth.[37] Soybean proteins complement the proteins of grains, such as rice, corn, and wheat that have lysine as their limiting amino acid, in order to provide a diet in which all of the essential amino acids are provided in sufficient amounts.[9,37]

Table IV
Amino Acids Used by the Human Body[36]

Essential Amino Acids	Nonessential Amino Acids
Isoleucine	Alanine
Leucine	Arginine
Lysine	Asparagine
Methionine	Aspartic Acid
Phyenlalanine	Cysteine
Threonine	Glutamic Acid
Tryptophan	Glutamine
Valine	Glycine
	Proline
	Serine
	Tyrosine
	Histidine

Recommended Dietary Allowances (RDA) are a set of nutrient standards for a healthy human established by the United States government. The RDA for protein is 0.8 grams of protein per kilogram of appropriate body weight per day.[37] For example, a healthy 73-kilogram person (160 pounds) needs 58 grams of protein per day. The Food Guide Pyramid suggests that adults should consume two to three servings of protein-rich foods and two to three servings of dairy products per day.[7] Following Food Guide Pyramid suggestions, 58 grams of protein can be obtained from two 3-ounce servings of

meat and three 8-fluid ounce servings of 1% cow's milk, Table V.[39] However, 58 grams of protein can also be obtained with two 1-cup servings of boiled soybeans and three 8-fluid ounce servings of soymilk, Table V.[39]

Table V compares the protein and fat content of a 1-cup serving of boiled mature soybeans and a 3.5-ounce serving of beef prime rib.[29] The serving sizes of soybeans and beef prime rib represent standard amounts. Each product provides nearly 50% of the daily protein requirement of a healthy 73-kilogram person. The fat content of the soybean serving provides approximately five grams less fat than obtained from a serving of beef prime rib.[39]

Table V
Protein and Fat Content of Comparable Soy and Non-soy Foods[39]

Product	Protein, grams	Fat, grams
Mature soybeans, boiled; 250 ml (1 cup); 172 grams	28.6	15.4
Beef Prime Rib, roasted with 1/4" fat trim; 3.5 ounces; 100 grams	26.7	21.1
Soy milk 250 ml (8 fluid ounces)	6.6	4.6
2% Cow's Milk 250 ml (8 fluid ounces)	8.1	4.7
1% Cow's Milk 250 ml (8 fluid ounces)	8.0	2.6

Source: Pennington.[39]

Consumption of 8-fluid ounces of soy milk supplies approximately 1.5 grams less protein than either 1% or 2% cow's milk.[39] However, the fat content of an 8-fluid ounce serving of soy milk is similar to that of an 8-fluid ounce serving of 2% cow's milk.[39] The protein and fat content of soy milk is significant when considering the importance of fat

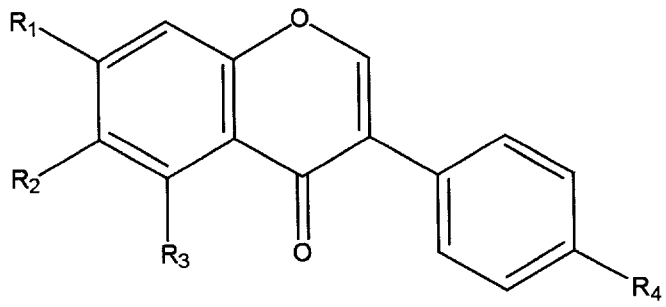
and protein in the diet of children being raised as vegetarians. Though supplying less protein that 1% or 2% cow's milk, soy milk provides adequate amounts of both protein and fat essential for periods of rapid growth experienced by children.[37]

Soybean Isoflavones

In addition to the quality protein found in soybeans, this food source is recognized for its high content of isoflavones.[11] Soybean isoflavones are categorized as phytoestrogens.[40] In a simplified manner, phytoestrogens are estrogens from phyto, or plant sources. The chemical structure of isoflavones is similar to the human estrogen hormone.[41]

Song et al. has compiled data on 12 isoflavone isomers.[24] Nine of the isomers are glucosides: genistin, daidzin, glycitin, acetylgenistin, acetyldaidzin, acetylglycitin, malonylgenistin, malonyldaidzin, and malonylglycitin.[24] Three of the isoflavone isomers exist as aglycone in soyfood with genistein being most abundant (64%), followed by daidzein (23%) and glycitein (13%).[12,24]

Due to the similarity of structure to human estrogen, soybean isoflavones have been shown to bind to estrogen receptors in the body.[40,41] The binding of soybean isoflavones to human estrogen receptors becomes particularly important because the amount of free estrogen in the circulatory system is low, representing less than 5% of the total estrogen in the body.[42] Unlike human estrogen, however, soybean isoflavones do not have a high binding affinity to serum proteins, albumin, or sex hormone binding globulin.[42]



Isoflavone	R_1	R_2	R_3	R_4
Genistin	*O*-glucosyl	H	OH	OH
Genistein	OH	H	OH	OH
Daidzin	*O*-glucosyl	H	H	OH
Daidzein	OH	H	H	OH
Glycitin	*O*-glucosyl	OCH_3	H	OH
Glycitein	OH	OCH_3	H	OH

Figure 2. Structure of the Isoflavones Genistein, Daidzein, and Glycitein and their glucosides Genistin, Daidzin, and Glycitin[12]

Genistein and Genistin

Naim et al. have determined that isoflavones represent 0.25% of the weight of soybeans.[12] The soybean isoflavone of particular interest is genistein, the primary isoflavone found in soybeans.[11] Soybeans are one of the few foods that contain a substantial amount of the isoflavone genistein.[43] Genistein is the aglycone form of the soybean isoflavone genistin. The chemical name of genistein is 5,7,4'-trihydroxyisoflavone.[44]

Genistin is more abundant than its aglycone genistein in soybeans. Naim et al. found that the majority of the isoflavones (99%) in soybeans are present in their glucoside form.[12] Genistin has the same chemical structure as genistein except genistin has a glucose molecule that is chemically bonded to it through a beta-linkage.[11] The chemical name of genistin is 5,7,4'-trihydroxyisoflavone-7-glucoside.[44] Walter determined that the glucose molecule attached to genistin is *d*-glucose.[44]

Digestion of Genistein

In the intestine, a ß-glucosidase cleaves the glucose molecule from genistin to form genistein, as shown in Figure 3.[42] The amount of genistein formed from its glucoside depends upon the composition of the diet and the amount and kind of microflora present in the intestines.[42,45] A significant amount of genistein is formed by bacterial action in the gut.[17]

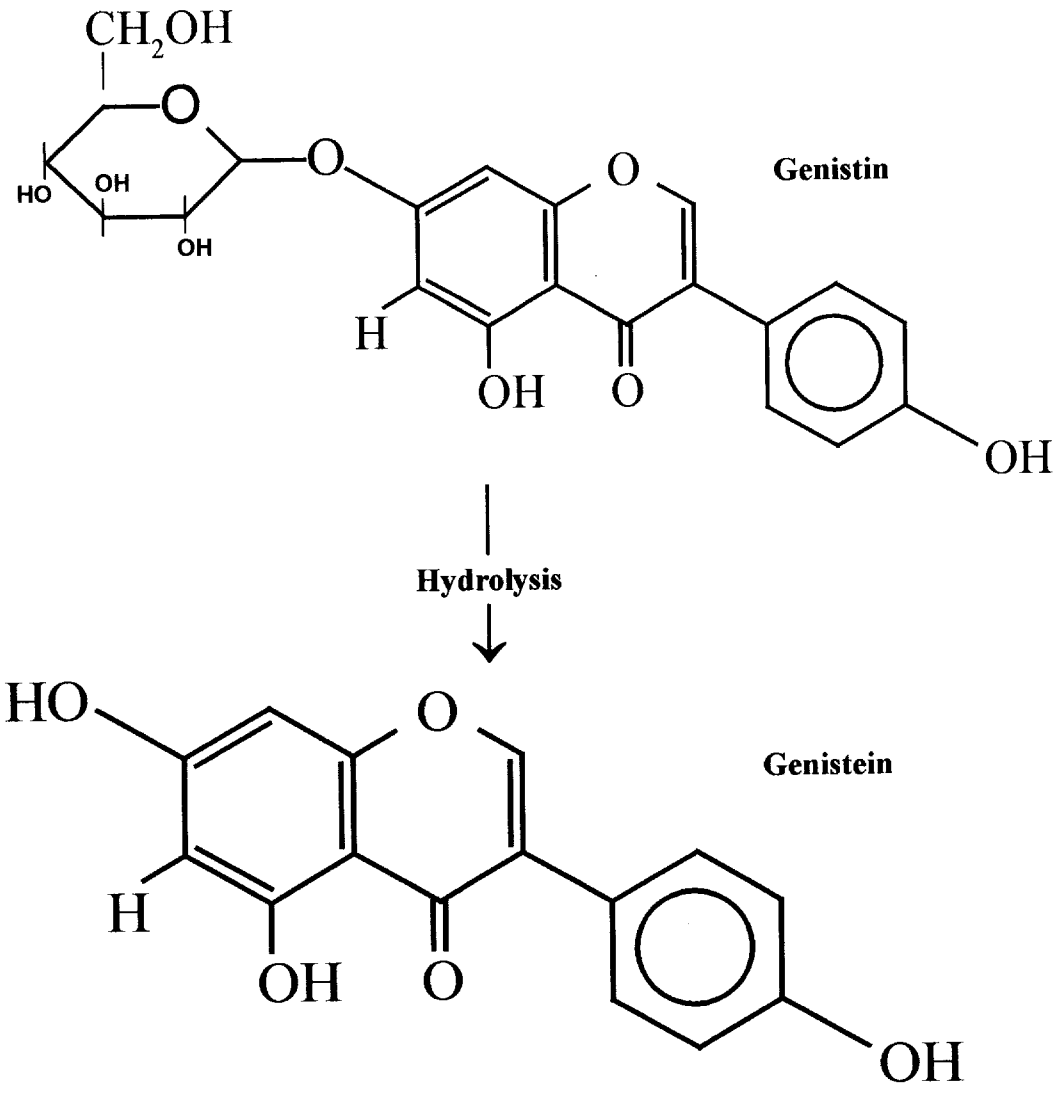


Figure 3. The Hydrolysis of Genistin to Genistein

Absorption of Genistein

Genistein and genistin have been found to be highly bioavailable in rats.[46] Sfakianos et al. found that genistein was well absorbed in the intestine and then traveled through an enterohepatic cycle from the intestine, to the portal blood, to the liver, into the bile, and back to the intestine.[46] King et al.[47] found that the initial rate of absorption is greater when genistein is ingested in its aglycone form compared to the initial rate of absorption when genistein is ingested in its glucoside form genistin. King et al.[47] found, however, that although genistein-fed rats initially had higher plasma concentrations of genistein at 2 hours post-feeding and greater urinary excretions of genistein at 2 and 8 hours post-feeding than genistin-fed rats, no significant difference was found in plasma concentrations of genistein at 8, 15, and 24 hours post-feeding of both groups and in urinary excretion of genistein at 48 hours post-feeding of both groups. In addition, no significant difference was found in fecal excretion of isoflavones by genistein- and genistin-fed rats.[47] Song et al.[24] reported that the genistein content in food should be normalized, meaning that the amount of genistein present in the glucoside form should be mathematically manipulated in order to compensate for the glucosides that would be hydrolyzed into aglycones in the gut before absorption.

Processing Effects on Genistein

In addition to changes in isoflavone composition brought about in the digestive system, soybean-processing methods, such as heating, change the relative amounts of genistin and genistein.[8,25,28,48] The amount of genistein in soybeans is also influenced by climatic growing conditions as well as the variety of the soybean.[24,32] Because of differences in genistein levels in soybeans based on their variety and growing conditions,

comparisons of levels of genistein and genistin among different soy products or sources may not be accurate. Comparative analyses of a particular soyfood product prepared identically should be performed in order to obtain accurate comparisons of isoflavone content.[24] Therefore, the data shown in Table VI should be used only as a guideline to the amount of genistein and genistin in soyfoods.

The amount of genistein is greater in the soy products that have undergone certain types of additional processing, Table VI. New soybeans, which have undergone only the harvesting process, contain a low concentration of genistein.[49] Coward et al.[28] noted that grinding soybeans into flour had no effect on the level of genistein present. Soymilk and

Table VI
Concentrations of Genistin and Genistein (μg/g) dry weight in Soybeans and other Soyfoods[49]

Soy Product	Genistein, (μg/g) dry weight	Genistin, (μg/g) dry weight
New Soybeans [a]	15.3	2413
Soy Grits	19	2080
Roasted Soybeans [b]	71.3	1830
Toasted Soybeans [c]	19.0	2080
Tofu [d]	138	1420
Tempeh [e]	224	1007

[a] Soybeans were harvested in October 1993, from a farm in Menomonie, WI.
[b] Product of Mother Nature's Foods, Eau Claire, WI.
[c] Fearn Soya Granules, product of Fearn Natural Foods, Milwaukee, WI.
[d] Extra firm silken tofu, product of Morinaga Nutritional Foods Inc., Torrance, CA.
[e] Tempeh Delites, product of Harvest Earth Foods, Inc., Rochester, MI.

Source: Vande Linde, AMQ, Tsui E, Unpublished data.[49]

tofu, which undergo a soaking process and hot aqueous extraction, contain substantially more genistein than the new soybeans, but mainly contain the ß-glucosides.[24,28] Fermented soybean products, such as tempeh, miso, and natto, have more of the aglycone genistein than nonfermented soybean products, such as tofu and soymilk.[24,27,48] Due to

its high solubility, genistein is removed from a soybean product when aqueous alcohol processing methods are used.[25,48] In the preparation of soy protein concentrate, an extraction process with a hot aqueous ethanol solution may be used, which results in a soy protein concentrate that is almost devoid of all isoflavones.[28]

Soybean Glucosidases

Matsurra et al.[50] determined that ß-glucosidases present in soybeans enzymatically hydrolyze the glucose molecule from genistin to form genistein during the soaking step of tofu preparation. Table VII shows the change in genistin and genistein content after the soybeans have been soaked in water for 16 hours at 20 °C.[50] In comparison to the isoflavone daidzin, genistin was more readily hydrolyzed by the ß-glucosidase enzymes present in soybeans.[51]

Table VII
Genistin and Genistein Amounts before and after Soaking in Water for 16 hours at 20 °C[50] *

Soybeans	Genistin (mg/100g dry weight)	Genistein (mg/100g dry weight)
Before soaking	181.1	4.9
After soaking	119.8	24.3

* Average of two replicates. Means within the same column are significantly different ($p \leq 0.05$).

The action of ß-glucosidases is time dependent, which means that more of the aglycone genistein was formed as time of contact increased.[51] Optimum conditions for hydrolyzing genistin to genistein by the ß-glucosidases have been established to be a pH of 5.5 and a temperature of 45 °C.[51] Data from a study by Matsurra & Obata[51] and an earlier study by Matsurra et al.[50] established that the optimum conditions for ß-glucosidase activity were a pH of 6.0 and a temperature of 50 °C. The action of the ß-

glucosidases can be inhibited by D-glucono-δ-lactone and by mercury (II) chloride ($HgCl_2$).[50,51] The inhibition of ß-glucosidase enzymes during processing can reduce the off-flavor that is produced by the production of the aglycone genistein.[50]

Tempeh

Tempeh is a fermented soybean product that undergoes a sequence of soaking, cooking, inoculation, and incubation/fermentation.[4,52] Tempeh originated in the Java regions of Indonesia.[53] Tempeh is a popular food among all socio-economic groups throughout the country of Indonesia.[53] Vegetarians consume tempeh because it is a good source of protein, which can substitute for meat in the diet. One serving of tempeh (83 grams = 1/2 cup) contains 15.7 grams of protein.[39] Tempeh is also a good source of dietary fiber and dietary calcium.[39] Because tempeh is a plant-source protein, tempeh contains no cholesterol.[39] Table VIII shows a partial breakdown of the nutrient composition of tempeh.[39]

Table VIII
Nutritional Content of Tempeh[39]

	Calories (kcal)	Protein (g)	Fat (g)	Carbohydrate (g)	Calcium (mg)	Iron (mg)
Tempeh; 1/2 cup; (83 g)	165	15.7	6.4	14.1	77	1.88

Source: Pennington[39]

The amount of the aglycone genistein in tempeh is well documented to be greater than that found in non-fermented soyfoods.[24,25,27,28] The fermentation process is suggested to be the cause of the greater aglycone genistein content.[25,27]

Tempeh Mold - *Rhizopus oligosporus*

Rhizopus oligosporus is the mold responsible for the formation of tempeh. Classified under the Kingdom Fungi, the mold *Rhizopus oligosporus* is a nonphototrophic, heterotrophic absorber.[54] After a batch of cooked soy grits is cooled, a powdered tempeh starter is added that contains *Rhizopus oligosporus*. During incubation, *Rhizopus oligosporus* produces a white mycelium throughout the soy grits, resulting in a firm, unified, white tempeh cake.

Health Benefits of the Isoflavones Genistein and Genistin

Research interest in isoflavones from soybeans is not new. However, the number of research articles on the subject has grown dramatically within the last ten years. Because isoflavones are biologically active in the body, Whitten[55] concluded that each isoflavone should be categorized individually based upon its effectiveness or lack thereof.[55]

Genistein has been found to have fungistatic,[12] antioxidative,[12,56] antimutagenic,[14] and diuretic properties.[15] The fungistatic properties of isoflavones are primarily expressed by the aglycone form. The glucoside forms of genistein, daidzein, and glycitein were not as powerful fungistatic agents as the aglycone forms of the isoflavones.[12] The *in vitro* antioxidative properties of isoflavones are influenced by the number of hydroxyl groups present on the molecules; the addition of a glucose molecule to the aglycone decreased the antioxidative property of those isoflavones.[12,56] Genistein has a greater antimutagenic property than daidzein.[14] A hydroxyl group present on carbon-5 of the genistein molecule may be responsible for its greater antimutagenic property compared to daidzein, which has a hydrogen atom on carbon-5.[14] In an *in vitro*

study, genistein induced significant vasorelaxation, caused increased diuresis, natriuresis, and kaluresis, and acted as a loop diuretic by inhibiting the Na^+-K^+-$2Cl^-$ cotransport, which can be helpful in the treatment of kidney and heart diseases.[15]

Genistein has reported anticarcinogenic properties.[57] The mechanism by which genistein acts as an inhibitor of tumor growth is not understood.[57] Because genistein has a chemical structure similar to the female human estradiol, genistein can bind to human estrogen receptors.[41] Santell et al.[19] determined that the relative binding affinity of genistein to the estrogen receptor to be approximately one percent of that of estradiol.

Genistein may have protective effects against development of breast, mammary, and prostate cancers.[16-18] In a study of immature female rats, Lamartiniete et al.[16] found that pharmacological doses of genistein enhanced mammary gland differentiation and had protective effects against chemically induced mammary cancer. Peterson et al.[17] found *in vitro* that genistein can inhibit tumor growth in the early stages of breast cancer. However, higher levels of genistein, perhaps in pharmacological levels, may need to be administered for the treatment of clinically diagnosed breast cancer.[17]

Human feeding studies with soybean products have been conducted with concurrent monitoring of the urinary excretion of isoflavones.[45,58-62] Xu et al.[45] suggested that the amount of the urinary excretion of isoflavones is directly related to the amount of isoflavones ingested. Hutchins et al.[62] found that the average urinary genistein recovery was 13% from subjects fed a soybean pieces diet containing 881 micrograms genistein per serving whereas the average urinary genistein recovery was 19% from subjects fed a tempeh diet containing 7265 micrograms per serving. Adlercruetz et al.[63] found a high

urinary excretion of isoflavones by Japanese men and women consuming a traditional Japanese diet, which contains a large amount of soybean products.

Treatment with genistein may help cancer patients. Genistein and genistein glucosides as well as daidzein glucosides were found to assist in the inhibition of cancer progression *in vitro* by (1) competing with endogenous estrogen for estrogen receptors thereby affecting the proliferation of estrogen-dependent cancer cells and (2) boosting the immune system through the activation of natural killer cells by a mechanism different from that of interleukin-2, which is another promoter of natural killer cells.[20]

Genistein may be able to help stop foreign intestinal bacterial infections in immuno-compromised patients. In an *in vitro* study, genistein-treated intestinal cells (enterocytes) were able to inhibit internalization of the foreign intestinal bacteria *Listeria monocytogenes*, *Salmonella typhimurium*, *Proteus mirabilis*, and *Escherichia coli* in a dose-dependent manner.[21] Genistein can not only be useful for the treatment of total body bacterial infections brought about by exposure to foreign intestinal bacteria in immuno-competent patients but also and can be used to prevent such infections in immuno-compromised patients who are more susceptible to foreign intestinal bacterial infections.[21] The high doses used in this study may be difficult to obtain from the diet; therefore, pharmacological doses may need to be given to obtain the effect against foreign intestinal bacteria in immuno-compromised patients.[21]

The effectiveness of genistein exposure during the lifetime of women is not fully understood. Santell et al.[19] hypothesized that perhaps genistein would be more effective in post-menopausal women rather than pre-menopausal women, particularly for the necessary promotion of estrogen-receptive tissues. Using a rat model to simulate

menopause, ovariectomized rats fed genistein experienced growth of estrogen-receptive tissues.[19] Lamartiniere et al.[22], however, hypothesized that genistein would be more effective in the early stages of life. Using a rat model, Lamartiniere et al.[22] found that genistein had beneficial effects on tumor growth in postpartum genistein-treated rats with chemically-induced with mammary cancer.[22] In that study, genistein-treated rats were given a dose of genistein on Day 2, 4, and 6 postpartum and then on Day 50 were given the mammary cancer inducer dimethylbenz[a]anthracene (DMBA).[22] The genistein-treated rats had an 88% incidence of mammary tumors in comparison to a 100% incidence in the vehicle-only-treated rats.[22]

Lamartiniere et al.[22] found that genistein promoted early cell differentiation in mammary glands that resulted in fewer terminal end buds, which are more susceptible to chemical carcinogens.[22] As terminal end buds evolve to lobules, the susceptibility of rat mammary glands to chemical carcinogens decreased. The differentiation of the mammary cells is the basic mechanism by which these cells are protected from the action of these chemical carcinogens.[22] At Day 21, the genistein-treated rats had significantly fewer terminal end buds and terminal ducts than the vehicle-only-treated rats.[22] At Day 50, the genistein treated rats had significantly fewer terminal end buds than the vehicle-only-treated rats.[22]

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is simply abbreviated as HPLC. HPLC has been used by many researchers for the determination of the genistein content of soyfoods.[24,25,27-29,64-67] HPLC is a superior form of chromatography because of its simplicity of operation and its relatively short analysis time.

An HPLC system has seven major components:

- Automated Gradient Controller
- Solvents
- Pumps
- Automatic Injector
- Column
- Detector
- Computer Data Processing System

The automated gradient controller is used to store specifications for an HPLC program designed to analyze particular components. In an HPLC system involving solvent concentration gradients, there are usually two pumps and two solvents. Each pump regulates the flow of one type of solvent. A pump can be set to have a particular flow rate. If an automated gradient controller is used, the automated gradient controller regulates the proportion of each solvent by regulating the flow rate of each pump regardless of the settings on the pumps.

An automated injector can be set to select a particular injection volume, number of samples, and run time. Usually the automated injector has a carriage that holds many HPLC sample vials. After the injection is made, the solvent mixture containing the injected sample flows through the column before detection by a detector.

HPLC is a type of chromatography that is used to separate components dissolved in a solution. Solvents containing the sample are forced through a separation column by the high pressures used in HPLC. The separation columns used in HPLC vary in length, internal diameter, composition, and size of the internal packing particles. The columns

used in HPLC are usually quite expensive, ranging from 100 to 300 dollars. Therefore, in order to protect the column, a guard column is placed in-line prior to the separation column. The guard column serves to filter out any particles from the sample that may obstruct the separation column.[68]

The elution of the components of a solution is dependent upon the polarity of the components and the type of phase used in HPLC. There are two types of phases used in HPLC: normal-phase and reverse-phase. Normal-phase chromatography has a polar stationary phase (column packing material) and a nonpolar mobile phase (solvent system). In normal-phase chromatography, the highly polar components of a sample would elute only after the nonpolar components and the less polar components have eluted from the column. Reverse-phase chromatography has a nonpolar stationary phase (column packing material) and a polar mobile phase (solvent system). In reverse-phase chromatography, the highly polar components of a sample would elute first, followed by the less polar components and the nonpolar components of the sample.

There are two types of analytical procedures in HPLC. An isocratic procedure uses the same solution or ratio of solutions throughout the entire analysis of the sample. A gradient procedure, however, uses a ratio of the solutions that is varied throughout the analysis of the sample. An automated gradient controller is used to program a gradient analytical procedure. A gradient procedure was used by Fukutake et al.[27], Coward et al.[25,28], Eldridge [64], and Song et al.[24] in the analysis of the isoflavone composition of soybean products.

The detectors vary in HPLC analysis. The choice of the detector may be based on effectiveness of the detector and/or the availability of a detector. Fukutake et al.[27] and

Song et al.[24] used a photodiode array detector to detect isoflavones extracted from soybean products. Coward et al.[25,28] used mass spectrometry to detect isoflavones extracted from soybean products. In another study, Coward et al.[25] used proton nuclear magnetic resonance spectroscopy (P-NMR) for detection of these compounds. A photodiode array detector was used in this study. The information from the detector was sent to a computer equipped with a data processing system. The data processing system processed the information from the detector and produced a chromatogram with processed peak areas and peak heights.

CHAPTER III

METHODOLOGY

Facilities

This research project was conducted at the Department of Chemistry and the Department of Food and Nutrition, University of Wisconsin-Stout, Menomonie, WI.

Materials and Reagents

All of the necessary chemicals (solvents, reagents, and equipment) were purchased from Fisher Scientific (Fair Lawn, NJ). The isoflavone standards (genistin and genistein) were purchased from Indofine Chemicals Inc. (Somerville, NJ). HPLC grade methanol was used. Milli-Q water, water filtered with a micropore filtering system, was used for the preparation of solvents and samples used in the HPLC analytical procedures. Certified organic soy grits (Great River Organic Milling, Inc., Winona, MN) were purchased from the Menomonie Food Co-op. The acetic acid was Gedney's White Distilled Vinegar (Chaska, MN), which had $5\%_{vol}$ acidity. Lactic acid, 85%, Food Chemicals Codex (FCC) (Aldrich Product Number: W261106) and the Citric acid, 99.5+%, FCC (Aldrich Product Number: W230618) were purchased from the Flavors and Fragrance Division of Sigma-Aldrich Chemical Company (St. Louis, MO).

Instrumentation

A High-Performance-Liquid-Chromatograph (HPLC) equipped with a Millipore/Waters model 710A WISP auto-injector and two Millipore/Waters model M6000A pumps was used in this study. A Millipore/Waters model 440 dual channel absorbance detector was interfaced with an Macintosh Apple IIvx computer equipped with Dynamax MacIntegrator Version 1.4.3 software (Rainin Instrument Company, Inc.).

A Discovery C18 column (250 mm x 4.6 mm; 5 micron-diameter beads of amorphous silica with dimethyl octadecylsilyl bonded to its surface) Supelco (Bellefonte, PA) was used. Instrument conditions and parameters for the detection of the isoflavones at ambient temperature were:

- Mobile phases: methanol and 0.10-M ammonium acetate buffer, pH 4.60
- Flow rate: 0.80 mL/minute
- Injection Volume: 20 microliters
- Detection at 254 nm.

Procedure

Figure 4 illustrates the steps in the experimental procedure for the preparation of tempeh.

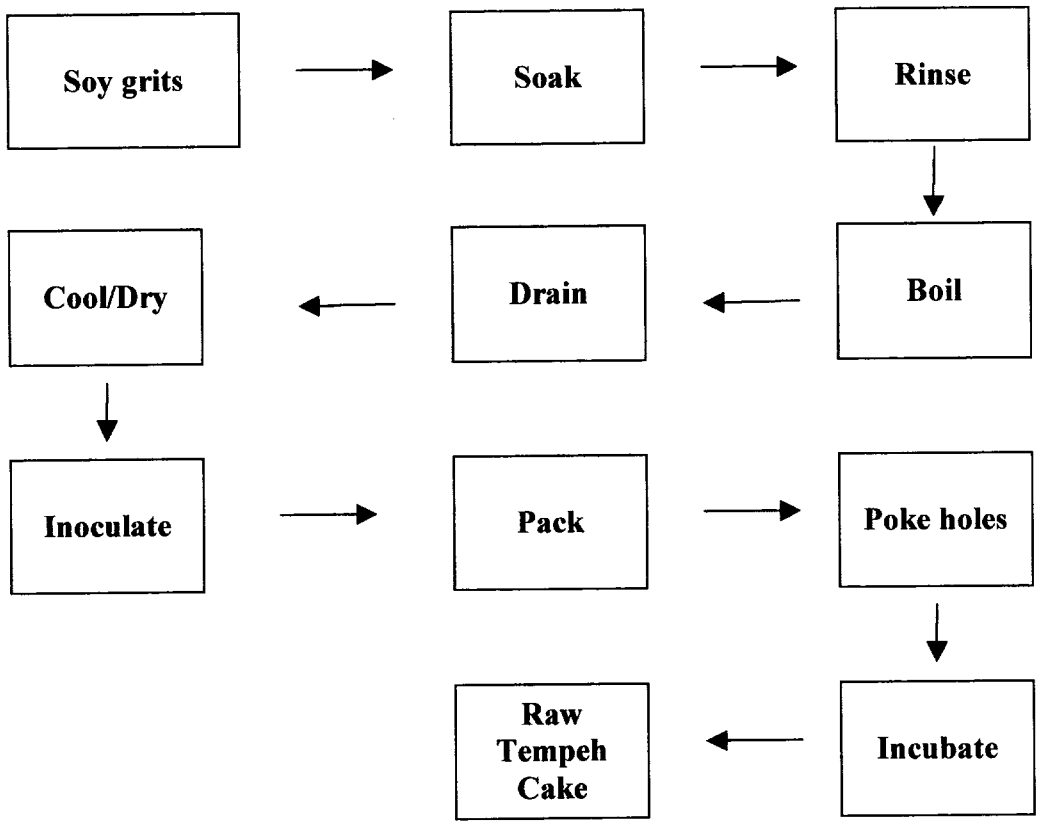


Figure 4. Flow Chart of Tempeh Preparation

Initial Material

Soy grits were used in this study. Soy grits are soybeans that are lightly toasted and then cracked into small pieces.[69] The flavor attributes and the nutritional value of soy grits and soybeans are similar.[69] The cooking time for soy grits is shorter than for whole soybeans, which is attributed to the difference in size.[69]

Soaking, Cooking, Inoculation, and Incubation

Table IX lists the parameters under which the soy grits were processed in the making of tempeh in this study. Individual 125 mL quantities of dry soy grits were soaked in 500 mL of residential tap water or in a $0.1\%_{vol}$ solution of lactic acid, $0.1\%_{mass}$ solution of citric acid, or $0.1\%_{vol}$ solution of acetic acid, Figure 5, for either one or two hours. The soaked soy grits were then rinsed with 250 mL of fresh soaking solution. The soaked and rinsed soy grits were placed in a 1–quart stainless steel cooking pot containing 850 mL of a cooking solution. The cooking solution was either water or a $0.1\%_{vol}$ solution of lactic acid, $0.1\%_{mass}$ solution of citric acid, or $0.1\%_{vol}$ solution of acetic acid. An electric range (Sears Robuck, Inc.) was used to bring the suspension to a rolling boil. Cooking time was initiated as soon as a rolling boil was observed. The soy grits were boiled for 40 minutes, Figure 6. Bits of soybean hulls were skimmed from the top of the suspension with a slotted spoon as needed during the cooking period to prevent the solution from boiling over the pan.

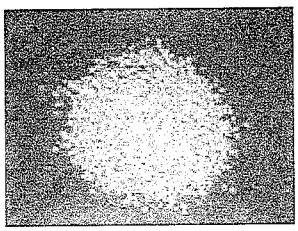


Figure 5. Soy Grits before Soaking

Figure 6. Soy Grits Cooking

Table IX

Parameters under which Soybean Grits were Processed to Make Tempeh

Sample *	Soaking Solution	Soaking Time, Hours	Cooking Solution
Water/1/Water (Control)	Water	1	Water
Water/2/Water (Secondary Control)	Water	2	Water
Lactic/1/Water	$0.1\%_{vol}$ Lactic Acid	1	Water
Lactic/2/Water	$0.1\%_{vol}$ Lactic Acid	2	Water
Water/1/Lactic	Water	1	$0.1\%_{vol}$ Lactic Acid
Water/2/Lactic	Water	2	$0.1\%_{vol}$ Lactic Acid
Lactic/1/Lactic	$0.1\%_{vol}$ Lactic Acid	1	$0.1\%_{vol}$ Lactic Acid
Lactic/2/Lactic	$0.1\%_{vol}$ Lactic Acid	2	$0.1\%_{vol}$ Lactic Acid
Citric/1/Water	$0.1\%_{mass}$ Citric Acid	1	Water
Citric/2/Water	$0.1\%_{mass}$ Citric Acid	2	Water
Water/1/Citric	Water	1	$0.1\%_{mass}$ Citric Acid
Water/2/Citric	Water	2	$0.1\%_{mass}$ Citric Acid
Citric/1/Citric	$0.1\%_{mass}$ Citric Acid	1	$0.1\%_{mass}$ Citric Acid
Citric/2/Citric	$0.1\%_{mass}$ Citric Acid	2	$0.1\%_{mass}$ Citric Acid
Acetic/1/Water	$0.1\%_{vol}$ Acetic Acid	1	Water
Acetic/2/Water	$0.1\%_{vol}$ Acetic Acid	2	Water
Water/1/Acetic	Water	1	$0.1\%_{vol}$ Acetic Acid
Water/2/Acetic	Water	2	$0.1\%_{vol}$ Acetic Acid
Acetic/1/Acetic	$0.1\%_{vol}$ Acetic Acid	1	$0.1\%_{vol}$ Acetic Acid
Acetic/2/Acetic	$0.1\%_{vol}$ Acetic Acid	2	$0.1\%_{vol}$ Acetic Acid

* All sample names represent the parameters under which the soy grits were processed to make tempeh. For example, Water/1/Water is a tempeh made with water soaking solution, soaked for 1 hour, and a water cooking solution.

After the cooking period, the soy grits were drained using a sieve, which was shaken to facilitate cooling and drying of the soy grits. The damp soy grits were spread on a wire cooling rack covered by 3 or 4 layers of cheesecloth until the soy grits were cool to the touch and free of all beads of liquid, Figure 7. Additional cheesecloth was placed over the soy grits and patted gently to facilitate the drying and cooling processes. The soy grits were transferred to a medium-sized stainless steel mixing bowl. After measuring approximately 1/8-teaspoon (approximately 0.5 mL) of Powdered Tempeh

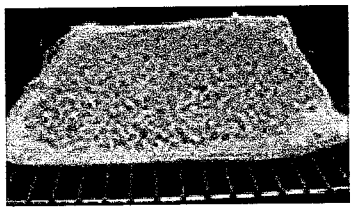


Figure 7. Soy Grits Drying and Cooling

Starter (G.E.M. Cultures, Fort Bragg, CA) containing the mold *Rhizopus oligosporus*, half of the Powdered Tempeh Starter (approximately 0.25 mL) was added to the soy grits, Figure 8. A stainless steel spoon was used to distribute the starter evenly among the soy grits. The remaining portion (approximately 0.25 mL) of the Powdered Tempeh Starter was added and, again, the soy grits were thoroughly mixed in order to facilitate even distribution of the starter.

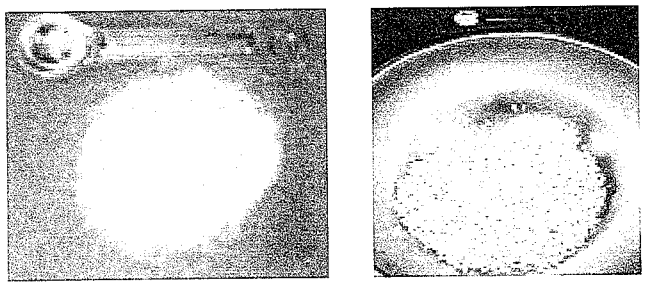


Figure 8. Cooled Soy Grits ready for Inoculation

The inoculated soy grits were lightly, yet firmly, packed into a 6-1/2" x 2-3/4" resealable plastic snack bag (Ziploc®, S.C. Johnson, Racine, WI). The bag was sealed and the contents were distributed evenly throughout the bag, obtaining a final thickness of approximately 1/4- to 1/2-inch. The bag was placed on a wire rack and a clean sewing needle was used to puncture holes in a grid-like pattern, 1/4- to 1/2-inch apart, on the top

side of the bag. The bag was firmly, yet gently, compressed to expel any excess air and to facilitate sufficient grit-to-grit contact. The appropriately labeled bag of inoculated soy grits was incubated in a biological incubator (Modern Lab Equipment of New York, Model Number 205-ss) at 31 +/- 2 °C for 22.5 hours, Figure 9. After incubation, the raw tempeh cake was removed from the incubator and prepared for storage, Figure 10.

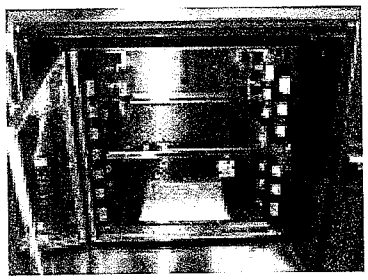


Figure 9. Incubation of Packaged Soy Grits

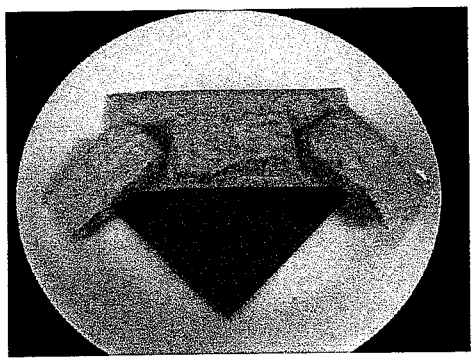


Figure 10. Raw Tempeh Cake

Tempeh Samples

Since each tempeh sample was prepared in triplicate, three trials for each of the tempeh parameters were produced.

Storage

When the raw tempeh cake was removed from the incubator after its 22.5 hour incubation period, the removal time and date was recorded. The bag containing the raw tempeh cake was wrapped in plastic wrap (Saran Wrap, S.C. Johnson, Racine, WI) and placed in a resealable freezer bag (Ziploc, S.C. Johnson, Racine, WI). The bag was placed in an upright freezer, being careful not to stack the tempeh cakes until the contents were completely frozen in order to prevent continued mold growth. The frozen raw tempeh cake remained in the freezer at -17 °C until freeze-drying.

Freeze-drying

A frozen raw tempeh cake was removed from the plastic bag and broken into 8-15 pieces to facilitate the process of thoroughly freeze-drying the tempeh. LabConco Corporation (Kansas City, MO) freeze-drying equipment was used with a LabConco freezer dryer (Catalog No.: 75033). After a dual layer of filter paper (LabConco Corp P/N: A-75448) was placed in the outlet hole of rubber top for the freeze-dryer beaker, the bent-glass arm was attached to the rubber top. The rubber top was placed securely on the 600-mL freeze-dryer beaker containing the pieces of tempeh. After the machine was turned on, two freeze-dryer beakers were attached to two ports on the freeze-dryer. The freeze-drying process required 24 hours at a temperature below –40 °C. The freeze-dried tempeh was place in a 32-ounce glass jar, covered with plastic wrap (Saran Wrap, S.C.

Johnson, Racine, WI), and frozen in a typical household refrigerator freezer at -17 °C until the grinding was to take place.

Grinding

The freeze-dried tempeh cake was ground to a powder consistency by a Commercial Bar Mixer/Blender (Hamilton Beach/Proctor-Silex, Inc.). The ground tempeh was stored in a plastic specimen cup, labeled, and placed in a typical household refrigerator freezer at -17 °C until preparation for HPLC.

HPLC Sample Preparation

A portion of the freeze-dried frozen tempeh was transferred to a ceramic pestle and ground to a fine powder by hand with a ceramic grinder. Each trial of a tempeh sample was prepared in triplicate. Using a calibrated Mettler/Toledo B303 scale (Switzerland), 0.500 grams of tempeh powder was massed in a 1 5/8" x 1 1/4" x 5/16" plastic pour boat weighing dish (Fisher #02-204-1A). The tempeh powder was quantitatively transferred from the weighing boat to a 50-mL glass-stoppered Erlenmeyer flask with a total of 10 mL of HPLC grade, filtered 80% methanol. The flask was stoppered, and placed in a 60 °C shaking water bath agitated at 160 cycles/minute for 1 hour.

After the one hour, the sample suspension was removed from the water bath and allowed to cool to room temperature. The sample suspension was vacuum-filtered through a Millipore ground-glass filter and a 25-mm glass fiber filter (Gelman Sciences: Ann Arbor, MI; P/N: 61630). The filtrate was quantitatively transferred to a 10-mL glass volumetric flask (KIMAX, Type A) using 80% methanol. The flask was diluted to volume with 80% methanol, stoppered, and mixed by inversion 10 times. This flask contained the tempeh extract that was used for HPLC analysis.

Each HPLC vial, cap, and rubber diaphragm was rinsed thoroughly with 80% methanol and allowed to dry (approximately 5 to 10 minutes). A small portion of the tempeh extract was poured into the vial, shaken to rinse the entire surface of the vial, and discarded. The vial was then filled at least half full with the tempeh extract solution. The vial was capped, labeled, and placed in the HPLC sample carriage.

HPLC Buffer Preparation

In the preparation of the HPLC Buffer, 7.7 grams of HPLC-grade Ammonium Acetate salt (MCB Reagents: Cincinnati, OH) was massed into a 250-mL beaker. Approximately 60 mL of Milli-Q water was added to the beaker. The contents were mixed until the salt had dissolved. Ten mL of HPLC-grade 12 M Glacial Acetic Acid (Fisher Scientific: Fair Lawn, NJ) was added. The solution was then diluted with Mill-Q water to a volume of 100. mL and mixed thoroughly.

A 1000-mL graduated cylinder was filled with 700 mL of Milli-Q water. Then 78.8 mL of the Ammonium Acetate salt/Glacial Acetic Acid solution was added and mixed. The solution was diluted to a final volume of 1000 mL with Milli-Q water and mixed again.

A magnetic stirrer was placed in the graduated cylinder, which was placed on a magnetic stirring plate. The pH of the solution was established using a calibrated Sargent Welsh 6050 pH meter (Skokie, IL). The pH was adjusted to 4.6 with 15 M NH_4OH or 12 M Glacial Acetic Acid. The buffer was vacuum-filtered through a 47-mm glass fiber filter (Gelman Sciences: Ann Arbor, MI; P/N: 64798) supported on a Millipore ground-glass filter. The filtered buffer was placed in a 1000-mL brown glass chemical storage jug and labeled appropriately.

HPLC Methanol Preparation

HPLC-grade methanol (Fisher) was vacuum-filtered through with a Millipore ground-glass filter supporting a 47-mm glass fiber filter (Gelman Sciences: Ann Arbor, MI; P/N: 64798). The filtered methanol was placed in a 1000-mL brown glass chemical storage jug and label appropriately.

80% Methanol Preparation

In a 1000-mL graduated cylinder, 800 mL of HPLC grade methanol and 200 mL of Milli-Q water were mixed. The solution was vacuum-filtered through a Millipore ground-glass filter supporting a 47-mm glass fiber filter (Gelman Sciences: Ann Arbor, MI; P/N: 64798). The filtered 80% methanol was placed in a 1000-mL brown glass chemical storage jug and label appropriately.

HPLC Analysis

Prior to the analysis of samples, the HPLC was allowed to operate in the isocratic mode (25% methanol/75% buffer (vol/vol)) for approximately 30 minutes. The HPLC procedure used to analyze the extracts of the tempeh samples and the isoflavone standards was a 70-minute program, Table X. A sample injection volume of 20 µl was used during HPLC analysis. Initially, a linear solvent gradient (Curve 6) was established, changing the composition of the solvent system from 25% methanol/75% buffer (vol/vol) to 60% methanol/40% buffer (vol/vol) over 20 minutes followed by an isocratic interval of 25 minutes during which 60% methanol/40% buffer (vol/vol) was the solvent system. After that, a second linear gradient (Curve 6) was established, changing the solvent system back from 60% methanol/40% buffer (vol/vol) to 25% methanol/75% buffer

(vol/vol) over 5 minutes. The initial conditions of75% buffer/25% methanol (vol/vol) continued for the next 20 minutes to complete the 70-minute program.

Table X
HPLC Analysis 70-minute Program

Time	Flow Rate	Volume, % Methanol	Volume, % Buffer	Curve
Initial	0.8 mL/min.	25	75	*
20	0.8 mL/min.	60	40	6
45	0.8 mL/min.	60	40	6
50	0.8 mL/min.	25	75	6
70	0.8 mL/min.	25	75	6

Genistin Standard Stock Solution

A genistin stock solution was prepared from a genistin isoflavone standard (Indofine Chemicals Inc.; Somerville, NJ; Claimed Purity = 100%). Subsequent to weighing 0.100 g of pure solid genistin, the sample was quantitatively transferred with 80% methanol into a 10-mL glass volumetric flask (KIMAX, Type A), diluted to volume with 80% methanol, and mixed well by inversion.

The genistin standard solution was used to prepare a series of diluted genistin standards. Precise volumes of the genistin stock solution were transferred into 10-mL glass volumetric flasks (KIMAX, Type A) and diluted to volume with 80% methanol. These diluted genistin standards were mixed well by inversion and used for HPLC calibration. The retention time of the pure genistin in the diluted stock solutions established the peak retention time for identifying the genistin peak on chromatograms of analyzed tempeh extract solutions.

Genistein Standard Stock Solution

A genistein stock solution was made from a genistein isoflavone standard (Indofine Chemicals Inc.; Somerville, NJ; Claim Purity = 100%). Subsequent to weighing 0.150 g of pure solid genistein, the sample was quantitatively transferred with 80% methanol into a 10-mL glass volumetric flask (KIMAX, Type A), diluted to volume with 80% methanol, and mixed well by inversion.

The genistein standard solution was used to prepare a series of diluted genistein standards. Precise volumes of the genistein stock solution were transferred into 10-mL volumetric flasks (KIMAX, Type A), diluted it to volume with 80% methanol, and mixed well by inversion. Table XII shows the various dilutions of the genistein standard solution used for HPLC peak calibration. Data collected from HPLC analysis of the diluted genistein standard solutions were used to develop a standard curve (Results, Figure 12). That standard curve was used to determine the amount of genistein in the tempeh samples. The retention time of pure genistein in the diluted stock solutions established the peak retention time for identifying genistein on chromatograms of analyzed tempeh extract solutions.

Retention Time Verification through Spiking

A tempeh extract was prepared and divided into three portions. One portion of the extract was spiked with the genistin stock solution and analyzed by HPLC in order to determine the retention time of genistin. Another portion of the extract was spiked with the genistein stock solution and analyzed by HPLC in order to determine the retention time of genistein. The final portion of the extract was not spiked and was analyzed by HPLC. The genistin-spiked tempeh extract was used to verify the location of the genistin

peak in the chromatogram of the tempeh extract. The genistein-spiked tempeh extract was used to verify the location of the genistein peak in the chromatogram of the tempeh extract.

Data Retrieval

The areas (μV-sec) of the genistein peaks were recorded for each tempeh sample. By using the genistein standard curve, peak areas were converted into concentrations of genistein in milligrams of genistein per gram tempeh dry weight.

Conversion of Sample Peak Area to Genistein Concentration

Step 1. Sample Peak Area: 1,484,937 μV-sec; from the chromatogram.

Step 2. Best-fit Line Equation from Genistein Standard Curve:

$$y = 482070x + 48278.$$

Step 3. Solve for the x-value to determine Genistein Concentration.

X-value = 3.00 mg of genistein/g tempeh dry weight.

Data Analysis

An F-test[70] was performed to determine if the standard deviations of two mean values were significantly different. If the standard deviations of the mean values were found to have no significant difference, then a Student's T-test[70] was performed. If the standard deviations of the mean values were found to have a significant difference, then the standard deviations were evaluated for errors.

A Student's T-test[70] was performed in order to determine if two mean values were significantly different at a particular confidence level.

CHAPTER IV

RESULTS

Qualitative Identification of Genistin and Genistein

The retention times of the isoflavones genistin and genistein were determined by HPLC chromatography of standard solutions of the pure compounds. Genistin eluted at 18 minutes while genistein eluted at 29 minutes. In the tempeh samples, peak identification was done by spiking a sample with a genistin standard solution and a genistein standard solution. A single tempeh sample was analyzed three times; one without spiking, Figure 11A and 11C, one spiked with the genistin standard solution, Figure 11B, and one spiked with the genistein stock solution, Figure 11D.

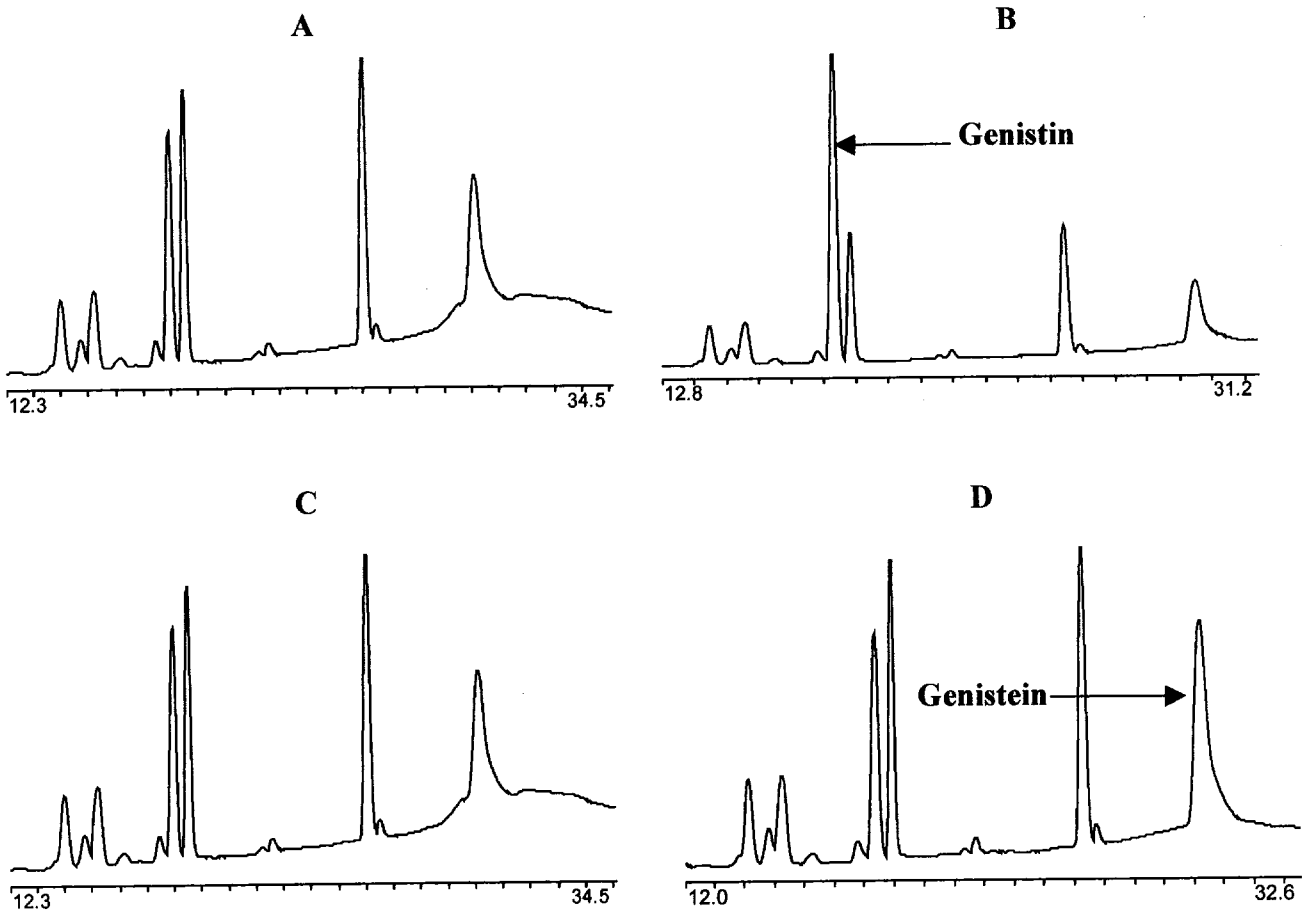


Figure 11. A and C – Chromatogram of Unspiked Tempeh Sample, B – Chromatogram of Genistin Spiked Sample, D – Chromatogram of Genistein Spiked Sample

Figure 11B shows that the area of the peak that eluted around 18 minutes was greater than the same peak shown in Figure 11A. This confirmed that this peak was genistin.

Figure 11D shows that the area of the peak that eluted around 29 minutes was greater than the same peak shown in Figure 11C. This confirmed that this peak was genistein.

Quantitative Determination

Different concentrations of the genistein standard solutions were used to construct a standard curve for genistein. Peak areas of the isoflavone genistein standard solutions are shown in Table XI.

Table XI

Peak Areas of the Genistein Standard Solutions

Genistein Concentration (mg/g dry weight)+	**Peak Area, μV-sec**
1.00 mg/g	623,260
2.00 mg/g	973,687
3.00 mg/g	1,484,937
4.00 mg/g	1,981,513
5.00 mg/g	2,457,261

+ Unit: milligram genistein per gram tempeh dry weight.

The standard curve for genistein was developed using the data collected from the peak areas and concentrations of the diluted standard solutions for genistein. This curve was generated using a graphing program KaleidaGraph (Synergy Software) and is shown in Figure 12. The best-fit line equation for the data points was

$$y = 482070x + 48278.$$

The correlation coefficient of the line was 0.99846. The best-fit line equation was used for the calculation of the amount of genistein in the tempeh samples.

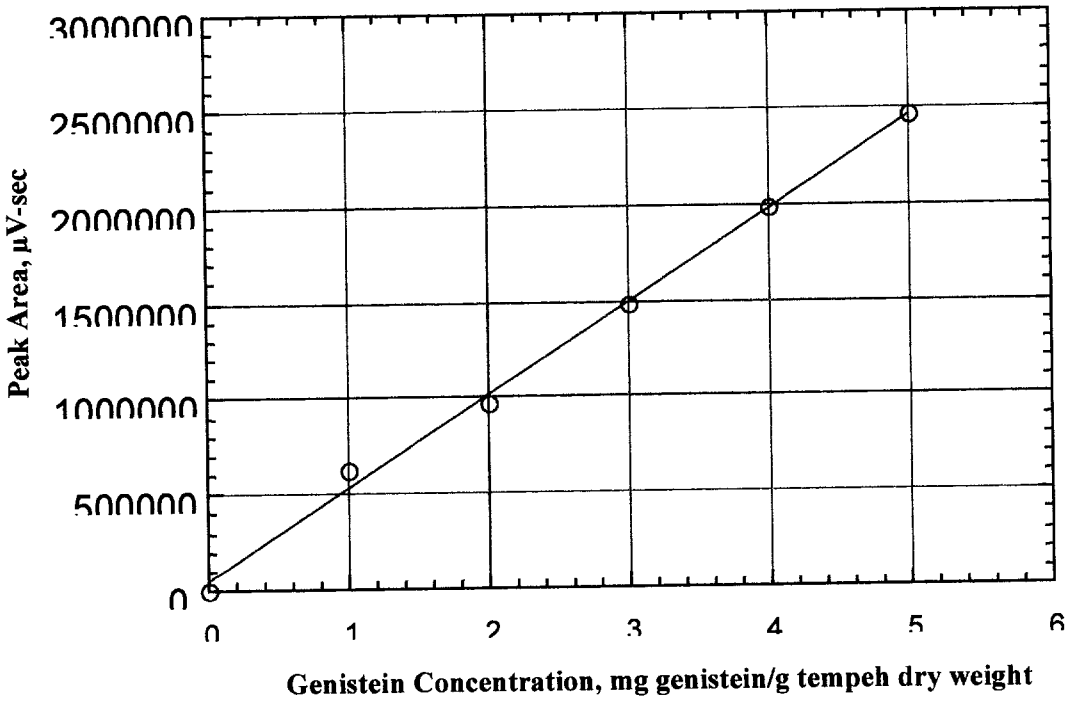


Figure 12. Genistein Standard Curve

Concentration of Genistein in Soy Grits and Tempeh

Table XII shows the concentration of genistein (mg/g dry sample) in soy grits and the control tempeh. The control tempeh (Water/1/Water) was a tempeh produced with a one-hour water soak and water as the cooking solution.

Table XII

Concentration (mg/g dry sample) of Genistein in Soy Grits and Tempeh

Sample	Genistein Concentration (mg/g dry weight)+
Water/1/Water	49.70 ± 3.64 *
Soy Grits	7.08 ± 1.94

Water/1/Water – one-hour soak with water; water cooking solution
+ Unit: milligram genistein per gram tempeh dry weight.
* Significantly greater than soy grits ($p < 0.001$).

Concentration of Genistein in Tempeh Samples

Table XIII shows the effect of a one- and two-hour soaking time on the final concentration of genistein in tempeh prepared by these procedures. The mean genistein concentration (mg/g dry sample) was 49.70 in the Water/1/Water tempeh and was 46.33 in the Water/2/Water tempeh. In this project, Water/2/Water was used as a secondary control for those tempehs that had a two-hour soaking period.

Table XIII

Effect of the Duration of Soaking Time on the Concentration Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/1/Water	49.70 ± 3.64
Water/2/Water	46.33 ± 7.47

+ Unit: milligram genistein per gram tempeh dry weight.
Water/1/Water – one-hour soak with water; water cooking solution - Control
Water/2/Water – two-hour soak with water; water cooking solution – Secondary Control

Table XIV shows the effects of various acidic soaking solutions on the final concentration of genistein in tempeh prepared by these procedures. The concentrations of genistein in Water/1/Water, Lactic/1/Water, Citric/1/Water, and Acetic/1/Water were 49.70, 24.83, 28.96, and 43.21 mg/g dry sample, respectively.

Table XIV

Effect of Various Acidic Soaking Solutions on the Concentration of Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/1/Water	49.70 ± 3.64
Lactic/1/Water	24.83 ± 7.69 *
Citric/1/Water	28.96 ± 2.58 *
Acetic/1/Water	43.21 ± 10.49

+ Unit: milligram genistein per gram tempeh dry weight.
Water/1/Water – one-hour soak with water; water cooking solution - Control
Lactic/1/Water – one-hour soak with $0.1\%_{vol}$ lactic acid; water cooking solution
Citric/1/Water – one-hour soak with $0.1\%_{mass}$ citric acid; water cooking solution
Acetic/1/Water – one-hour soak with $0.1\%_{vol}$ acetic acid; water cooking solution
* Significantly lower than TE 1 ($p < 0.05$)

Table XV shows the effects of various acidic soaking solutions with a 2-hour soak on the final concentration of genistein in tempeh prepared by these procedures. The concentrations of genistein in Water/2/Water, Lactic/2/Water, Citric/2/Water, and Acetic/2/Water were 46.33, 26.32, 30.55, and 40.99 mg/g dry sample, respectively.

Table XV

Effect of a 2-hour Soak in Various Acidic Soaking Solutions on the Concentration of Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/2/Water	46.33 ± 9.14
Lactic/2/Water	26.32 ± 5.29
Citric/2/Water	30.55 ± 2.08
Acetic/2/Water	40.99 ± 16.61

+ Unit: milligram genistein per gram tempeh dry weight.
Water/2/Water – two-hour soak with water; water cooking solution – Secondary Control
Lactic/2/Water – two-hour soak with $0.1\%_{vol}$ lactic acid; water cooking solution
Citric/2/Water – two-hour soak with $0.1\%_{mass}$ citric acid; water cooking solution
Acetic/2/Water – two-hour soak with $0.1\%_{vol}$ acetic acid; water cooking solution

Table XVI shows the effects of various acidic cooking solutions on the final concentration of genistein in tempeh prepared by these procedures. The concentrations

of genistein in Water/1/Water, Water/1/Lactic, Water/1/Citric, and Water/1/Acetic were 49.70, 31.95, 26.69, and 31.99 mg/g dry sample, respectively.

Table XVI

Effect of Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/1/Water	49.70 ± 4.46
Water/1/Lactic	31.95 ± 2.81 *
Water/1/Citric	26.69 ± 3.16 *
Water/1/Acetic	31.99 ± 8.32

+ Unit: milligram genistein per gram tempeh dry weight.
Water/1/Water – one-hour soak with water; water cooking solution – Control
Water/1/Lactic – one-hour soak with water; $0.1\%_{vol}$ lactic acid cooking solution
Water/1/Citric – one-hour soak with water; $0.1\%_{mass}$ citric acid cooking solution
Water/1/Acetic – one-hour soak with water; $0.1\%_{vol}$ acetic acid cooking solution
* Significantly lower than TE 1 ($p < 0.05$)

Table XVII shows the effect of various acidic cooking solutions following a 2-hour water soak on the final concentration of genistein in tempeh prepared by these procedures. The concentrations of genistein Water/2/Water, Water/2/Lactic, Water/2/Citric, and Water/2/Acetic were 46.33, 34.63, 31.89, and 30.55 mg/g dry sample, respectively.

Table XVIII shows the effect of various acidic soaking solutions and acidic cooking solutions on the final concentration of genistein in tempeh prepared by these procedures. The concentrations of genistein in Water/1/Water, Lactic/1/Lactic, Citric/1/Citric, and Acetic/1/Acetic were 49.70, 29.60, 33.08, and 29.80 mg/g dry sample, respectively.

Table XIX shows the effect of various acidic soaking solutions with a 2-hour soak and acidic cooking solutions on the final concentration of genistein in tempeh prepared by these procedures. The concentration of genistein in Water/2/Water, Lactic/2/Lactic,

Citric/2/Citric, and Acetic/2/Acetic were 46.33, 36.97, 35.16, and 36.92 mg/g dry sample, respectively.

Table XVII

Effect of Various Acidic Cooking Solutions following a 2-hour Water Soak on the Concentration of Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/2/Water	46.33 ± 9.14
Water/2/Lactic	34.63 ± 3.60
Water/2/Citric	31.89 ± 0.77
Water/2/Acetic	30.55 ± 9.35

+ Unit: milligram genistein per gram tempeh dry weight.
Water/2/Water – two-hour soak with water; water cooking solution – Secondary Control
Water/2/Lactic – two-hour soak with water; $0.1\%_{vol}$ lactic acid cooking solution
Water/2/Citric – two-hour soak with water; $0.1\%_{mass}$ citric acid cooking solution
Water/2/Acetic – two-hour soak with water; $0.1\%_{vol}$ acetic acid cooking solution

Table XVIII

Effect of Various Acidic Soaking Solutions and Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Tempeh Sample	Genistein Concentration (mg/g dry sample)+
Water/1/Water	49.70 ± 4.46
Lactic/1/Lactic	29.60 ± 5.59 *
Citric/1/Citric	33.08 ± 3.18 *
Acetic/1/Acetic	29.80 ± 9.84

+ Unit: milligram genistein per gram tempeh dry weight.
Water/1/Water – one-hour soak with water; water cooking solution – Control
Lactic/1/Lactic – one-hour soak with 0.1% lactic acid; $0.1\%_{vol}$ lactic acid cooking solution
Citric/1/Citric – one-hour soak with 0.1% citric acid; $0.1\%_{mass}$ citric acid cooking solution
Acetic/1/Acetic – one-hour soak with 0.1% acetic acid; $0.1\%_{vol}$ acetic acid cooking solution
* Significantly lower than TE 1 ($p < 0.05$)

Table XIX

Effect of Various Acidic Soaking Solutions with a 2-hour Soak and Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Tempeh Sample	**Genistein Concentration (mg/g dry sample)+**
Water/2/Water	46.33 ± 9.14
Lactic/2/Lactic	36.97 ± 5.56
Citric/2/Citric	35.16 ± 3.02
Acetic/2/Acetic	36.92 ± 9.38

+ Unit: milligram genistein per gram tempeh dry weight.
Water/2/Water – two-hour soak with water; water cooking solution – Secondary Control
Lactic/2/Lactic – two-hour soak with 0.1% lactic acid; $0.1\%_{vol}$ lactic acid cooking solution
Citric/2/Citric – two-hour soak with 0.1% citric acid; $0.1\%_{mass}$ citric acid cooking solution
Acetic/2/Acetic – two-hour soak with 0.1% acetic acid; $0.1\%_{vol}$ acetic acid cooking solution

CHAPTER V

DISCUSSION

The hypothesis subtending this project is that preparation of tempeh in an acidic soaking and/or cooking medium would enhance the level of genistein in that tempeh compared to tempeh prepared in a neutral medium (Control). The control tempeh (Control: Water/1/Water) was a tempeh with a one-hour water soak and water as the cooking solution. A secondary Control tempeh, Water/2/Water, was used for comparison of those tempehs prepared with a two-hour soaking period. The Control tempeh (Water/1/Water) contained the highest concentration of genistein of all tempeh preparations studied. All of the tempeh samples were prepared using soy grits.

Comparison of Genistein Concentration in Soy Grits and Tempeh

Table XII shows the concentration of genistein in soy grits and the Control tempeh (Water/1/Water). The concentrations of genistein in soy grits and the Control tempeh (Water/1/Water) were 7.08 and 49.70 mg/g dry sample, respectively. The data showed that the concentration of genistein in tempeh was significantly higher ($p < 0.001$) than in the nonfermented soy grits.

Researchers have repeatedly observed that fermented soybean products, such as tempeh, contain a higher level of genistein than present in nonfermented soybean products, such as tofu and soymilk.[25-27] The Control tempeh (Water/1/Water), prepared by a one-hour water soak and water as the cooking solution, had a concentration of genistein that was seven times greater than that of nonfermented soy grits.

Effect of the Duration of Soaking Time on the Concentration of Genistein in Tempeh

Table XIII shows the effect a one- or two-hour soaking of soy grits in water on the final concentration of genistein in tempeh produced by these procedures. The duration of soaking of the soy grits did not significantly change the final concentration of genistein in tempehs prepared by these procedures.

Effect of a 1-hour Soak in Various Acidic Soaking Solutions on the Concentration of Genistein in Tempeh

Table XIV shows the effects of a one-hour soak of soy grits in various acidic soaking solutions on the final concentration of genistein in tempehs prepared by these procedures. The use of $0.1\%_{vol}$ lactic acid (Lactic/1/Water) and $0.1\%_{mass}$ citric acid (Citric/1/Water) soaking solutions significantly decreased ($p < 0.05$) the concentration of genistein in these tempehs compared to the Control tempeh (Water/1/Water). The use of $0.1\%_{vol}$ acetic acid soaking solution (Acetic/1/Water), however, did not have a significant effect on the concentration of genistein in that tempeh compared to the Control tempeh (Water/1/Water), which was prepared from soy grits soaked in water.

Effect of a 2-hour Soak in Various Acidic Soaking Solutions on the Concentration of Genistein in Tempeh

Table XV shows the effects of a 2-hour soak of soy grits in various acidic soaking solutions on the final concentration of genistein in tempeh prepared from those soy grits. The 2-hour soaking of soy grits in various acidic soaking solutions did not have a significant effect on the final concentration of genistein in those tempehs compared to the

tempeh prepared from soy grits soaked in water for 2 hours (Secondary Control; Water/2/Water).

Effect of Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Table XVI shows the effects of various acidic cooking solutions on the final concentration of genistein in tempehs prepared using this procedure. The $0.1\%_{vol}$ lactic acid (Water/1/Lactic) and $0.1\%_{mass}$ citric acid (Water/1/Citric) cooking solutions significantly decreased ($p < 0.05$) the concentration of genistein in the subsequently prepared tempehs compared to tempeh cooked in water (Control; Water/1/Water). However, the $0.1\%_{vol}$ acetic acid (Water/1/Acetic) cooking solution did not significantly affect the concentration of genistein in that tempeh compared to tempeh from soy grits cooked in water (Control; Water/1/Water).

Effect of Various Acidic Cooking Solutions with a 2-hour Water Soak on the Concentration of Genistein in Tempeh

Table XVII shows the effect of various acidic cooking solutions following a 2-hour water soak on the final concentration of genistein in tempehs prepared by this procedure. The acidic cooking solutions containing either $0.1\%_{vol}$ lactic acid, $0.1\%_{mass}$ citric acid, or $0.1\%_{vol}$ acetic acid following a 2-hour water soak (Water/1/Lactic, Water/2/Citric, & Water/2/Acetic) did not cause a significant change in the concentration of genistein in these tempeh preparations compared to tempeh prepared from soy grits soaked in water for 2 hours and cooked in water (Secondary Control; Water/2/Water).

Effect of Various Acidic Soaking Solutions with a 1-hour Soak and Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Table XVIII shows the effect of a 2-hour soak of soy grits in various acidic soaking solutions and acidic cooking solutions on the final concentration of genistein in tempeh made from soy grits prepared by this procedure. Tempeh prepared from soy grits treated with $0.1\%_{vol}$ lactic acid soaking and cooking solutions (Lactic/1/Lactic) and $0.1\%_{mass}$ citric acid soaking and cooking solutions (Citric/1/Citric) contained significantly less ($p < 0.05$) genistein compared to tempeh prepared from a 1-hour soak in water and cooking in water (Control; Water/1/Water). However, the $0.1\%_{vol}$ acetic acid soaking and cooking conditions (Acetic/1/Acetic) did not have a significant effect on the concentration of genistein in tempeh compared to the Control tempeh (Water/1/Water).

Effect of Various Acidic Soaking Solutions with a 2-hour Soak and Various Acidic Cooking Solutions on the Concentration of Genistein in Tempeh

Table XIX shows the effect of various acidic soaking solutions with a 2-hour soak and acidic cooking solutions on the final concentration of genistein in tempeh made from soy grits treated by these procedures. The acidic soaking solutions with a 2-hour soak and acidic cooking solutions (Lactic/2/Lactic, Citric/2/Citric, & Acetic/2/Acetic) did not significantly change the final concentration of genistein in these tempehs compared to tempeh prepared from soy grits soaked in water for 2 hours and cooked in water (Secondary Control; Water/2/Water).

CHAPTER VI

CONCLUSION

Based on the data collected in this project, the use of various acidic solutions in making tempeh did not significantly increase, but rather decreased, the final concentration of genistein in tempeh treated with acidic solutions. Therefore, the method recommended for producing tempeh includes the parameters followed for the control tempeh, Water/1/Water, which uses water as the soaking solution with a one-hour soak and water as the cooking solution. The control, Water/1/Water, contained the highest amount of genistein, 49.70 mg/g dry sample. The use of an acidic solution in the soaking period and/or the cooking period did not enhance the genistein concentration in tempeh.

Continuing Research

Continuing research in our laboratory is being conducted to explore the use of a ß-glucosidase enzyme. With the addition of the ß-glucosidase enzyme to the soaking solution, it is hypothesized that the glucose molecule attached to genistin would be cleaved, thus forming genistein.

Additional Research

Additional research should be conducted to determine whether or not the fermentation process is responsible for the increased level of genistein in tempeh. The methodology for this project would focus on the length of incubation of tempeh using the tempeh parameters that served as the Control tempeh in the present study, which are a one-hour soak and water as the cooking solution.

The standard incubation period of 22.5 hours that was used in the present study would serve as the Control incubation length in this suggested study. Extracts of soy

grits used for tempeh preparation would be analyzed by HPLC as they were in the present study to serve as a reference initial level of genistein in soy grits. In addition, soy grits would be analyzed just prior to incubation (after inoculation, but before packaging) to serve as a control tempeh with a zero hour incubation period. Removal of the tempeh would begin at 11.25 hours incubation (half of the control incubation period), 15 hours incubation, 18 hours incubation, 24 hours incubation, 28 hours incubation, 32 hours incubation, and 36 hours incubation. The premature removal of the tempeh would have insufficient mold growth and may appear as normal soy grits. The late removal of the tempeh would have sufficient mold growth and may show signs of sporulation by *Rhizopus oligosporus*, which is evident by the presence of black areas on the tempeh.

In total, 9 different tempehs would be produced, which includes the control. The tempehs would be grown in triplicate for a total of 27 individual tempehs. Each tempeh as well as the unprocessed soy grits would be analyzed by HPLC in triplicate using the HPLC methodology used in the present study. The final concentration of genistein in the tempehs would be compared to the concentration of genistein in a control tempeh (22.5 hours incubation) as well as in the unprocessed soy grits.

Depending on the results of the proposed additional research presented above, analysis of the soy grits during the various stages of tempeh production may be necessary to determine if the soaking and cooking stages of tempeh production affect the final concentration of genistein in tempeh. In this study, soy grits would be analyzed before tempeh processing, after the one-hour soaking period, and after the 40-minute cooking period to determine the amount of change, if any, in the concentration of genistein in soy grits before the inoculation/incubation step in tempeh processing.

CHAPTER VII

REFERENCES

1. Greenlee R, Murray T, Bolden S, Wingo P. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7-33.

2. Persky V, Van Horn L. Epidemiology of soy and cancer: perspectives and directions. J Nutr 1995;125:709S-712S.

3. Messina M. Modern applications for an ancient bean: soybeans and the prevention and treatment of chronic disease. J Nutr 1995;125:567S-569S.

4. Herman C, Adlercreutz T, Goldin B, Gorbach S, Hockerstedt K, Watanabe S, Hamalainen E, Markkanen M, Makela T, Wahala K, Hase T, Fotsis T. Soybean phytoestrogen intake and cancer risk. J Nutr 1995;125:757S-770S.

5. Arjmandi B, Alekel L, Hollis B, Amin D, Stacewicz-Sapuntzakis M, Guo P, Kukreja S. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161-167.

6. Ashendel C. Diet, signal transduction and carcinogensis. J Nutr 1995;125:686S-691S.

7. Lee R, Nieman D. Nutritional Assessment. 2nd ed. St. Louis, MO: Mosby, 1996.

8. Messina M, Messina V. Increasing use of soyfoods and their potential role in cancer prevention. J Am Diet Assoc 1991;91:836-840.

9. Erdman J Jr, Fordyce E. Soy products and the human diet. Am J Clin Nutr 1989;49:725-37.

10. Golbitz P. Traditional soyfoods: processing and products. J Nutr 1995;125:570S-572S.

11. Messina M. Legumes and soybeans: overview of their nutritional profiles and health effects. Am J Clin Nutr 1999;70(suppl):439S-50S.

12. Naim M, Gestetner B, Zilkah S, Birk Y, Bondi A. Soybean isoflavones. Characterization, determination, and antifungal activity. J Agric Food Chem 1974;22:806-810.

13. Naim M, Gestetner B, Bondi A, Birk Y. Antioxidative and antihemolytic activities of soybean isoflavones. J Agric Food Chem 1976;24:1174-7.

14. Miyazawa M, Sakano K, Nakamura S-I, Kosaka H. Antimutagenic activity of isoflavones from soybean seeds (*Glycine max* Merrill). J Agric Food Chem 1999;47:1346-1349.

15. Martinez R, Gimenez I, Lou J, Mayoral J, Alda J. Soy isoflavonoids exhibit in vitro biological activities of loop diuretics. Am J Clin Nutr 1998;68(suppl):1354S-7S.

16. Lamartiniere C, Zhang J-X, Cotroneo M. Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity. Am J Clin Nutr 1998;68(suppl):1400S-5S.

17. Peterson G, Ji G-P, Kirk M, Coward L, Falany C, Barnes S. Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines. Am J Clin Nutr 1998;68(suppl):1505S-11S.

18. Aldercreutz H, Mazur W, Bartels P, Elomaa V-V, Watanabe S, Wahala K, Landstrom M, Lundin E, Bergh A, Damber J-E, Aman P, Widmark A, Johansson A, Zhang J-X, Hallmans G. Phytoestrogens and prostate disease. J Nutr 2000;130:685S-659S.

19. Santell R, Chang Y-C, Nair M, Helferich W. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypthalamic/pituitary axis in rats. J Nutr 1997;127:263-269.

20. Zhang Y, Song T, Cunnick J, Murphy P, Hendrich S. Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. J Nutr 1999;129:399-405.

21. Wells C, Jechorek R, Kinneberg K, Debol S, Erlandsen S. The isoflavone genistein inhibits internalization of enteric bacteria by cultured Caco-2 and HT-29 enterocytes. J Nutr 1999;129:634-640.

22. Lamartiniere C, Moore J, Brown N, Thompson R, Hardin M, Barnes S. Genistein suppresses mammary cancer in rats. Carcinogenesis 1995;16:2833-2840.

23. Murphy P, Song T, Buseman G, Barua K, Beecher G, Trainer D, Holden J. Isoflavones in retail and institutional soy foods. J Agric Food Chem 1999;47:2697-2704.

24. Song T, Barua K, Buseman G, Murphy P. Soy isoflavone analysis: quality control and a new internal standard. Am J Clin Nutr 1998;68(suppl):1474S-9S.

25. Coward L, Barnes N, Setchell K, Barnes S. Genistein, daidzein, and their ß-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. J Agric Food Chem 1993;41:1961-67.

26. Franke A, Hankin J, Yu M, Maskarinec G, Low S-H, Custer L. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. J Agric Food Chem 1999;47:977-986.

27. Fukutake M, Takahashi M, Ishida K, Kawamura H, Sugimura T, Wakabayashi K. Quantification of genistein and genistin in soybeans and soybean products. Food Chem Toxicol 1996;34:457-461.

28. Coward L, Smith M, Kirk M, Barnes S. Chemical modification of isoflavones in soyfoods during cooking and processing. Am J Clin Nutr 1998;68(suppl):1486S-91S.

29. Wang H-J, Murphy P. Isoflavone content in commercial soybean foods. J Agric Food Chem 1994;42:1666-1673.

30. Am J Clin Nutr 1998;68(suppl).

31. J Nutr 1995;125(suppl).

32. Smith A, Circle S. Historical background, in Soybeans: Chemistry & Technology, Vol 1, Smith A, Circle S, Eds., Westport, CT: AVI Publishing Co., 1978.

33. United States Department of Agriculture. 1997 Census of Agriculture: Selected Crops Harvested: 1997. http://www.nass.usda.gov/census/census97/highlights/ag-state.htm.

34. United Soybean Board. World Statistics: World Soybean Production 1998. http://www.unitedsoybean.org/99soystats/page_34.htm.

35. Woodruff S, Klass H. A study of soybean varieties with reference to their use as food. Univ Illinois Agr Expt Sta Bull 1938;433.

36. Groff J, Gropper S, Hunt S. Advanced Nutrition and Human Metabolism. *Chapter 7: Protein.* 2nd ed. St. Paul, MN: West Publishing Company, 1995.

37. Whitney E, Rolfes S. Understanding Nutrition. *Chapter 6: Protein: Amino Acids.* 6th ed. St. Paul, MN: West Publishing Company, 1993,

38. Liener. Chapter in Soybeans: Chemistry & Technology, Vol 1, Smith A, Circle S, Eds., Westport, CT: AVI Publishing Co., 1978.

39. Pennington J. Bowes and Church's food values of portions commonly used. 17th ed. Philadelphia: JB Lippincott, 1994.

40. Molteni A, Brizio-Molteni L, Persky V. In vitro hormonal effects of soybean isoflavones. J Nutr 1995;125:751S-756S.

41. Martin P, Horwitz K, Ryan D, McGuire W. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978;103:1860-1867.

42. Setchell K. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 1998:68(suppl):1333S-46S.

43. Price K, Fenwick G. Naturally occurring oestrogens in foods—a review. Food Addit Contam 1985;2:73-106.

44. Walter E. Genistin (an isoflavone glucoside) and its aglucone, genistein, from soybeans. J Am Chem Soc 1941;63:3273-6.

45. Xu X, Harris K, Wang H-J, Murphy P, Hendrich S. Bioavailability of soybean isoflavones depends upon microflora in women. J Nutr 1995;125:2307-2315.

46. Sfakianos J, Coward L, Kirk M, Barnes S. Intestinal uptake and biliary excretion of the isoflavone genistein in rats. J Nutr 1997;127:1260-1268.

47. King R, Broadbent J, Head R. Absorption and excretion of the soy isoflavone genistein in rats. J Nutr 1996;126:176-182.

48. Anderson R, Wolf W. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. J Nutr 1995;125:581S-588S.

49. Vande Linde AMQ, Tsui E. Unpublished data.

50. Matsuura M, Obata A, Fukushima D. Objectionable flavor of soy milk developed during the soaking of soybeans and its control. J Food Sci 1989;54:602-605.

51. Matsuura M, Obata A. ß-glucosidases from soybeans hydrolyze daidzin and genistin. J Food Sci 1993;58:144-147.

52. Shurtleff W, Aoyagi A. Tempeh Production. Lafayette, CA: Soyfoods Center, 1986.

53. Winarno F, Reddy N. Tempe in Legume-Based Fermented Foods. Reddy N, Pierson M, Salunkhe D, Eds., Boca Raton, FL: CRC Press, 1986.

54. Ingraham J, Ingraham C. Introduction to Microbiology. Belmont, CA: Wadsworth Publishing Company, 1995.

55. Whitten P, Lewis C, Russell E, Naftolin F. Potential adverse effects of phytoestrogens. J Nutr 1995;125:771S-776S.

56. Gyorgy P. Antioxidants isolated from fermented soybeans (tempeh). Nature 1964;203:870-2.

57. Peterson G. Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 1995;125:784S-789S.

58. King R, Bursill D. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. Am J Clin Nutr 1998;67:867-72.

59. Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, Arai Y, Mazur W, Wahala K, Adlercreutz H. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). J Nutr 1998;128:1710-1715.

60. Zhang Y, Wang G-J, Song T, Murphy P, Hendrich S. Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. J Nutr 1999;129:957-962.

61. Xu X, Wang H-J, Murphy P, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J Nutr 1994;124:825-832.

62. Hutchins A, Slavin J, Lampe J. Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. J Am Diet Assoc 1995;95:545-551.

63. Aldercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, Okada H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr 1991;54:1093-1100.

64. Eldridge A. High-performance liquid chromatography separation of soybean isoflavones and their glucosides. J Chromatogr 1982;234:494-496.

65. Setchell K, Welsh M-B, Lim C. High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. J Chromatogr 1987;386:315-323.

66. Wang G, Kuan S, Francis O, Ware G, Carman A. A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. J Agric Food Chem 1990;38:185-190.

67. Wang H-J, Murphy P. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 1994;42:1674-1677.

68. University of Kentucky College of Pharmacy. High Performance Liquid Chromatography (HPLC): A Users Guide. Accessed: 04AUG2000. http://kerouac.pharm.uky.edu/ASRG/HPLC/hplcmytry

69. Messina M, Messina V, Setchell K. The Simple Soybean and your Health. Garden City Park, NY: Avery Publishing Group Inc., 1994.

70. Day Jr. R, Underwood A. Quantitative Analysis. 6th Ed. Englewood Cliffs, NJ: Prentice Hall, 1991.